THE MICROTOMIST'S VADE-MECUM

(BOLLES LEE)

A HANDBOOK OF THE METHODS OF ANIMAL AND PLANT MICROSCOPIC ANATOMY

TENTH EDITION

EDITED BY

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PREFACE TO THE TENTH EDITION

In this new edition an important change has been made by the inclusion of plant technique. This section has been written by Dr. D. G. Catcheside, of London University, but has necessitated leaving out two sections previously included in the ninth edition. The extension of usefulness of the book which the inclusion of a section on plant technique will effect, should more than make up for this sacrifice. It is certain that the botanist or zoologist will benefit by looking over various chapters describing the techniques in the other sister science, and thus the new departure of putting both plant and animal micrology between the same two covers will have been justified.

New chapters now included are those on the frozen section technique, and on vital staining. The latter has been written by Dr. R. J. Ludford, whose great assistance and helpful advice throughout is gratefully acknowledged. In this edition, Dr. H. J. Conn, the leading American authority on staining, has recast the whole chapter on this subject, and Dr. E. S. Duthie has done the same for the article on blood and glands. Mr. K. C. Richardson has been responsible for the section on celloidin imbedding, and the chapter on tissue culture, and Dr. Kay and Dr. Whitehead have completely re-written the chapter on fats. Dr. Helen Pixell-Goodrich has again been responsible for the article on protozoological technique.

. Perhaps the most difficult section in the book is that devoted to the nervous system ; Dr. J. G. Greenfield and Dr. Ruby O. Stern undertook the necessary revision at considerable inconvenience to themselves. Their valuable collaboration has been especially helpful, and puts us deeply in their debt.

The ninth edition was going to press when the death of Arthur Bolles Lee was announced, and there was not time to make more than passing reference to his death, as well as to that of William Bayliss and of C. Da Fano, two previous contributors. It is fitting that some account of Arthur Bolles Lee should be given in this work. The Vade-Mecum appeared first in 1885, when he was thirty-six years of age, and during the time that he was an assistant at the Russian laboratory at Villefranche, near Nice. Lee subsequently brought out seven editions of his book, the last in 1913, when he was sixty-four years old. The eighth edition appeared in 1921, Lee sending the present senior editor 300 references. The ninth edition appeared in 1928. Lee was born at Coldrey House, in Hampshire, between Farnham and Alton, in 1849, and when he was eighteen he was articled to a London solicitor. On his mother's death in 1871 his inheritance enabled him to do as he liked —he broke his articles—became a medical student in London, but did not complete his course ; he drifted finally to Switzerland and attended

classes at the University of Neuchatel. He married a Swiss lady and spent the rest of his life in that country, dying at Clarens, in March, 1927. According to Dr. A. E. Boycott, of London University, who supplied the above facts, and has very kindly interested himself in the recent editions of the Vade-Mecum, Bolles Lee towards the end of his life became deeply engrossed in psychology and philosophical reading.

Between the recent editions and those of Bolles Lee there is this difference—Lee tried every method himself, but in recent years the subject has become so large, and the leisure of the collaborators and editors so small, that this desirable attribute has not been attained except in a few chapters. Several chapters in this book still stand almost as Bolles Lee wrote them—*e.g.*, those on invertebrate embryology and nerve endings.

The absence of Dr. Catcheside from England, and the fact that the editors, living so far apart, have had to depend on the written word for their collaboration, has made the editorship rather difficult, and it is with great pleasure that the kindness and patience of the publishers is acknowledged here.

To avoid further delay, it was not possible to submit this preface to the American co-editor. (This, however, gives the senior editor the opportunity of saying how great is his debt to Dr. Theophilus Painter. Not only has Professor Painter contributed a masterly article on animal chromosome technique, but he has helped very considerably in other parts of the book.) Both of us readily acknowledge the help we have received from various British and American colleagues. Among these must be mentioned Dr. E. A. Werner, Dr. K. C. Bailey and Dr. A. E. Werner, of the Dublin University Chemistry Laboratory, and Mr. Peter Gray, of the Edinburgh University Zoological Department. We also thank Dr. R. H. Micks and Mr. L. E. Werner for their contribution in the addendum. Finally, the index has been made by two Natural Science graduates of Trinity College, Dublin, Miss Olive Aykroyd and Miss Rosa Jones, whom we thank very cordially.

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PART I

CHAPTER I

INTRODUCTORY

1. The General Method. The methods of modern microscopic anatomy may be roughly classed as General and Special. There is a General or Normal method which consists in carefully *fixing* the structures to be examined, *staining* them with a *nuclear* stain, *dehydrating* with alcohol, and mounting *series of sections* of the structures in *balsam*. It is by this method that the work is planned and very often finished. Special points are then studied, if necessary, by Special Methods, such as examination of the living tissue elements, *in situ* or in " indifferent " media ; fixation with special fixing agents ; staining with special stains ; dissociation by teasing or maceration ; injection ; impregnation ; and the like.

There is a further distinction which may be made, and which may help to simplify matters. The processes of the preparation of tissues may be divided into two stages, *Preliminary Preparation* and *Ulterior Preparation*. Now the processes of preliminary preparation are essentially identical in all the methods, essential divergences being only found in the details of ulterior preparation. By preliminary preparation is meant that group of processes whose object it is to get the tissues into a fit state for passing unharmed through all the ulterior processes to which it may be desired to submit them. It comprehends the operations of (1) killing ; (2) fixing ; (3) the washing and other manipulations necessary for removing the fixing agent from the tissues, and substituting for it the preservative liquid or other reagents which it is desired to employ. Ulterior preparation comprehends the processes sketched out in §§ 3 *et seq.*

2. Preliminary Preparation. The first thing to be done with any structure is to *fix* its histological elements. (This statement applies equally to all classes of objects, whether it be desired to cut them into sections or to treat them in any other special way.) Two things are implied by the word " fixing " : first, the rapid *killing* of the element, so that it may not have time to change the form it had during life, but is fixed in death in

the attitude it normally had during life; and second, the *hardening* of it to such a degree as may enable it to resist without further change of form the action of the reagents with which it may subsequently be treated. *Without good fixation it is impossible to get good stains or good sections, or preparations good in any way.*

The structure, having been duly fixed by one of the processes described in the chapter on Fixing Agents, is, except in special cases, *washed* in order to remove from the tissues as far as possible all traces of the fixing reagent.

These operations having been duly performed, two roads become open. The object may be further prepared by what may be termed the *wet* method, in which all subsequent operations are performed by means of aqueous media. Or it may be further prepared by the *dehydration* method, which usually consists in treatment with successive alcohols of gradually increasing strength, final *dehydration* with absolute alcohol, imbibition with an essential oil or other so-called *clearing agent* which serves to remove the alcohol, and lastly either mounting at once in balsam or other resinous medium or imbedding in paraffin for the purpose of making sections. The dehydration method is the course which is generally preferred, chiefly because of its great superiority as regards the preservation of tissues. For the presence of water is the most important factor in the conditions that bring about the decomposition of organic matter, and its complete removal is the chief condition of permanent preservation.

3. Preservation. Considered as a mere dehydrating agent, *alcohol* fulfils its function fairly well (but see § 124). But considered as a histological *preservative* agent, it is far less satisfactory. If tissues be left in alcohol for only a few days before further preparation, injurious effects will perhaps not be very disagreeably evident. But it is otherwise if they are put away in it for many weeks or months before the final preparation is carried out. The dehydrating action of the alcohol being continuously prolonged, the minute structure of tissues is sometimes considerably altered by it; they become overhard and shrink, and become brittle, and their capacity for taking stains well becomes seriously diminished. Kultschitzky (*Zeit. wiss. Mik.*, iv, 1887, p. 349) has proposed to remedy this by putting up objects after fixation and washing out with alcohol in ether, xylol, or toluol. Flemming (*Arch. mik. Anat.*, xxxvii, 1891, p. 685) advises putting up objects after fixation in a mixture of alcohol, glycerin, and water, in about equal parts, pointing out that objects thus preserved may be at any moment either prepared for sectioning by treatment with pure alcohol or softened for dissection or teasing by a little soaking in water, and that they do not become so hard and brittle as alcohol specimens, and retain their staining power much better. Lee after extensive experience of this plan recommends it, and further

suggests that the action of the liquid seems to be in many cases much improved by addition of a little acetic acid (say 0·5 to 0·75 per cent.). (For Plants see § 1235.)

Prolonged treatment of tissues in alcohol is to be avoided whenever lipoid inclusions are being investigated. The higher strengths of alcohol and water have the power of bringing into solution many fatty substances which form the groundwork of the cytoplasmic inclusions. In addition, prolonged immersion in alcohol is fatal to success in most silver and gold impregnation methods of the neurologist. The so-called "hardening" effect of alcohol may be advantageous for rough work, but is better carried out in formalin or chrome salts.

For material that is intended *only for section-cutting*, it is undoubtedly by far the best plan to clear (next §) and imbed at once in paraffin. This affords an absolutely perfect preservation. Cedar-wood oil is nearly, if not quite, as good as paraffin, so far as the preservation of the tissues is concerned, but of course it is not so handy for storage.

4. Removal of Alcohol; Clearing. The water having been sufficiently removed, the alcohol is in its turn removed from the tissues, and its place taken by some anhydrous substance, generally an essential oil, which is miscible with the material used for imbedding or mounting. This operation is generally known as *Clearing*. It is very important that the passage from the last alcohol to the clearing agent be a *gradual* one. This is effected by placing the clearing medium *under* the alcohol. A sufficient quantity of alcohol is placed in a tube (a watch-glass will do, but tubes are generally better), and then with a pipette a sufficient quantity of clearing medium is introduced *at the bottom of the alcohol.* Or you may first put the clearing medium into the tube, and then carefully pour the alcohol on to the top of it. The two fluids mingle but slowly. The objects to be cleared, being now carefully put into the supernatant alcohol, float at the surface of separation of the two fluids, the exchange of fluids takes place gradually, and the objects slowly sink down into the lower layer. When they have sunk to the bottom, the alcohol may be drawn off with a pipette, and after some further lapse of time the objects will be found to be completely penetrated by the clearing medium. (Read also §§ 124 *et seq.*)

The clearing stage may be avoided by dioxan or N-butyl alcohol imbedding (see § 127).

This method of making the passage from one fluid to another applies to all cases in which objects have to be transferred from a lighter to a denser fluid—for instance, from alcohol, or from water, to glycerin.

This is a convenient stage for carrying out *minute dissections*, if any such have to be done, a drop of clearing agent being a most helpful medium for carrying out such dissections (see § 8).

At this point the course of treatment follows one of two different roads, according as the object is to be mounted direct in balsam or is first to be sectioned (§ 5).

5. Imbedding, and Treatment of Sections. The objects are now *imbedded.* They are removed from the clearing medium and soaked until thoroughly saturated in the imbedding medium. This is, for small objects, generally paraffin, liquefied by heat, and for large objects either paraffin or a solution of collodion or " celloidin " (in this last case the clearing may be omitted and the tissues be imbedded direct from the alcohol). The imbedding medium containing the object is then made to solidify, and sections are made with a microtome through the imbedding mass and the included objects. The sections are then mounted on a slide by one of the methods described in the chapter on Serial Section Methods, the imbedding material is removed from them (in the case of paraffin), they are stained *in situ* on the slide, dehydrated with alcohol, cleared, and mounted in balsam or damar. Or they may be stained, washed, dehydrated and cleared in watch-glasses, and afterwards mounted as desired—the imbedding medium being first removed if desirable.

Or, the material may be stained in bulk, before cutting the sections. In this case the object, after having been fixed and washed out, is taken from the water, or while still on its way through the lower alcohols (it should not be allowed to proceed to the higher grades of alcohol before staining, if that can be avoided), and passed through a bath of stain, then dehydrated with successive alcohols, passed through a clearing medium into paraffin, cut, and treated as above described, the sections in this case being mounted direct from the chloroform, xylol, or other solvent with which the paraffin is removed. If aqueous staining media be applied (and this is sometimes desirable), the structures should either be stained *in toto* immediately after fixing and washing out, or sections may be stained on the slide, the objects, if delicate, being passed through successive baths of alcohol of gradually decreasing strength before being put into the aqueous stain.

It is generally advisable not to stain in bulk material that is intended to be sectioned ; by staining it as sections the staining can be much better controlled, and many excellent stains can in this way be employed that are not available for staining in bulk ; and of course sections can be stained much more rapidly than material in bulk.

Balsam mounts of which the stain has faded, or which it may be desired to submit to some other staining process, or mount in some other medium, may often with great advantage be *re-stained* and *re-mounted.* All that is necessary is to put the slide into a tube of xylol or benzol till the cover falls off (about two days), wash well for

some hours in clean xylol, and pass through alcohol into the new stain. This succeeded in every case with series of sections mounted on Mayer's albumen, or by the water method. For shellac-mounted series, see E. Meyer, *Biol. Centralb.*, x, 1890, p. 509, or 7th *edition.*

The most convenient *vessels* in which to perform the various operations of staining, differentiating, dehydrating, clearing, etc., *on the slide*, are flat-bottomed corked glass tubes. Lee uses tubes 10 cm. high and 27 mm. internal diameter. Each of these will then take two slides, English size, placed back to back.

6. Résumé of the General Method. To sum up, you may either fix, wash out, stain, wash, dehydrate, clear, imbed, cut sections, clear and mount them in balsam; or fix, wash, dehydrate, clear, imbed, cut, stain, wash, dehydrate, clear and mount —according to choice. (See § 1424.)

7. Preparation of Entire Objects, or of Material that is not to be sectioned. The treatment of objects which can be studied without being cut into sections is identical with that above described, with the omission of those passages that relate to imbedding processes. Its normal course may be described as fixation, washing out, staining, treatment with successive alcohols of gradually increasing strength, final dehydration with absolute alcohol, clearing, and mounting in balsam.

In the *preparation of entire objects* or structures that are intact and *covered by an integument not easily permeable* by liquids, special care must be taken to avoid swelling from endosmosis on the passage of the objects from any of the liquids employed to a liquid of less density, or shrinkage from exosmosis on the passage to a liquid of greater density. This applies most specially to the passage from the last alcohol into the clearing medium. A slit should be made in the integument, if possible, so that the two fluids may mingle without hindrance. And in all cases the passage is made gradual by placing the clearing medium under the alcohol, as described (§ 4). Fluids of high diffusibility should be employed as far as possible in all the processes. Fixing agents of great penetrating power (each as picric acid or alcoholic sublimate solution) should be employed where the objects present a not easily permeable integument. Washing out is done with successive alcohols, water being used only in the case of fixation by osmic acid, or the chromic mixtures or other fixing solutions that render washing by water imperative. Staining is done by preference with alcoholic staining media. The stains most to be recommended are Grenacher's borax-carmine, or one of Mayer's alcoholic carminic acid or hæmatein stains. Aqueous stains are more rarely indicated, though there are many cases in which they are admissible, and some in which they are preferable.

8. Minute Dissections. These are best done, if necessary, in a drop of clearing agent. Lee recommends cedar-wood oil for this purpose as it gives to the tissues a consistency very favourable

for dissection, whilst its viscosity serves to lend support to delicate structures. Clove oil has a tendency to make tissues that have lain in it for some time very brittle. The brittleness is, however, sometimes very helpful in minute dissections. Another property of clove oil is that it does not easily spread itself over the surface of a slide, but has a tendency to form very convex drops, and this also makes it frequently a very convenient medium in which to make minute dissections.

If it be desired to dissect in a watery fluid, such as glycerin, it may be well to prepare the slide by spreading on it a thin layer of Mayer's albumen, and on this to place a small drop of glycerin, or other dissecting medium. As soon as the dissection has been accomplished, a cover may be let fall, horizontally, on to the preparation to keep the parts in place, and a weight placed on it. Then the mount may be filled up with glycerin, or other mounting medium, run in under the cover, and closed, if desired, or instead of the albumen a solution of gelatin may be taken, and hardened in formol with the objects on it. For a balsam mount, after clove or cedar oil, Schällibaum's collodion may be taken, and the organs fixed *in situ* on this by adding xylol.

9. **Microtomes** are instruments for the accurate production of thin slices of tissues. They are used both for cutting tissues that have acquired a certain favourable consistency through having been *imbedded* in *paraffin*, and also for cutting tissues that have been imbedded in softer masses, such as *collodion*, and tissues that have *not been imbedded* at all. Not all microtomes are equally well adapted for all these three classes of work. The microtome of the zoologist should at all events be one that is well adapted for cutting imbedded material.

Now there are two methods of imbedding in general use—the paraffin method and the celloidin method. In the paraffin method the object is cut *dry*, frequently with the knife set *square* to the line of section. In the celloidin method, as in the cutting of unimbedded tissues, it is generally cut *wet*, and always with a knife set *slanting*. Some microtomes that are well adapted for the paraffin method are ill adapted for the celloidin method or the cutting of unimbedded material, and *vice versâ*. It may be well to possess the two sorts of instrument; but if only one can be afforded it should be such as will give good work in either way.

Microtomes fall further into two classes according as the knife and the surface of section of the object are (A) in a horizontal plane, or (B) in a vertical plane. The former offer greater facility for the orientation of the plane of section, which is an important point for the zoologist and embryologist. Amongst these may be mentioned (*a*) The "Sliding" Microtomes, in which the knife is carried on a sledge and moved against the object. They work equally well with paraffin or celloidin, and some can be adapted as a freezing microtome. But this (as is the case with the others above mentioned) will not always furnish work of the highest accuracy; for the knife being only clamped at one end is liable to spring, and to give sections of unequal thickness. This

defect is remedied in (*b*), a type of sliding microtomes in which the knife is clamped at both ends and is a fixture, the object being carried on a sledge and moved against it.

Class A also includes some instruments in which the knife is carried on the horizontal arm and swung against the object by a rotary movement.

Class B contains instruments adapted for the production of continuous ribbons of sections by the paraffin method, but not so well adapted for celloidin or other work in the wet way, or for soft objects.

CHAPTER II

KILLING

10. In the majority of cases, the first step in the preparation of an organ or organism consists in exposing it as rapidly and as completely as possible to the action of one of the **Fixing Agents** that are discussed in the next chapter. The organ or organism is thus taken in the normal living state; the fixing agent serves to bring about at the same time, and with sufficient rapidity, both the death of the organism and that of its histological elements.

It should be noted that narcotisation generally implies some change in the cells, and most narcotics have to be applied for a long time. Such treatment is absolutely barred in material destined for careful cytological study. *This applies especially to ether and chloroform, which can be extremely injurious to cells:* in the case of larger mammals like the cat and the dog a preliminary treatment in ether or chloroform may be necessary, but directly after anæsthesia the animals' throats should be cut or they should be killed by a blow, if possible. Coal gas chambers are good for killing all mammals, because carbon monoxide does not appear to be hurtful to cells. Amphibians killed by chloroform are often completely spoilt for cytological purposes; if the brain is not wanted, pith the animal. For birds the time-honoured custom of wringing their necks is recommended. In the case of small lizards, newts and such live stock it is a good plan to cut off their heads quickly with strong scissors. If the material is wanted for chromosome or mitochondria work look up these sections for special directions.

But these methods are by no means applicable to all cases. There are many animals, especially such as are of a soft consistence, and deprived of any rigid skeleton, but possessing a considerable faculty of contractility, which if thus treated contract violently, and die in a state of contraction that renders them unfit for study. In these cases special methods of killing must be resorted to. Speaking generally, there are two ways of dealing with these difficult cases. You may kill the animal so suddenly that it has not time to contract: or you may paralyse it by narcotics before killing it. See also under "Chromosomes," § 621, and "Mitochondria," § 679.

SUDDEN KILLING

11. Heat. The application of *Heat* affords a means of killing suddenly. By it the tissues are more or less fixed at the same time that somatic death is brought about.

The difficulty consists in hitting off the right temperature, which is of course different for different objects. We think that 80° to 90° C. will generally be amply sufficient, and that very frequently it will not be necessary to go beyond 60° C. An exposure to heat for a few seconds will generally suffice.

Small objects (Protozoa, Hydroids, Bryozoa) may be brought into a drop of water in a watch-glass or on a slide, and heated over the flame of a spirit-lamp. For large objects, the water or other liquid employed as the vehicle of the heat may be heated beforehand and the animals thrown into it.

As soon as it is supposed that the protoplasm of the tissues is coagulated throughout, the animals should be brought into alcohol (30 to 70 per cent. alcohol) (if water be employed as the heating agent).

For frog's eggs, bring a large dish of water to the boil, remove bunsen and then drop in the egg masses ; remove after ten minutes into 50 per cent. alcohol (see also HERTWIG, § 829).

An excellent plan for preparing many marine animals is to kill them in *hot fresh water*. Some of the larger Nemertians are better preserved by this method than by any other with which we are acquainted.

12. Slowly Contracting Animals. Animals that contract but slowly, such as *Alcyonium* and *Veretillum*, and some Tunicates, such as *Pyrosoma*, are very well killed by throwing them into some very quickly acting fixing liquid, used either hot or cold. *Glacial* or very strong *acetic acid* (VAN BENEDEN's method) is an excellent reagent for this purpose ; it may be used, for example with some Medusæ. After an immersion of a few seconds or a few minutes, according to the size of the animals, they should be brought into alcohol of at least 50 per cent. strength. *Lemon juice* employed in this way has given Lee very good results with small Annelids and Hirudinea. *Corrosive sublimate* is another excellent reagent for this purpose.

NARCOTISATION

13. Narcotisation is performed by adding some anæsthetic substance very gradually, in very small doses, to the water containing the animals, and waiting patiently for it to take effect slowly.

PETER GRAY (WATSON'S *Microscope Record*, No. 35, 1935) says that for Protozoa he tries new forms with the following narcotics in this order : 10 per cent. methyl alcohol (not methylated), 1 per cent. hydroxylamine, 1 per cent. urethrane, Rousselet's solution, Corri's solution. " If these fail, hope may well be abandoned." For Stalked Ciliates he uses Rousselet's solution,

until the snapping movements have slowed up, then very gingerly, weak hydrogen peroxide. For *Planarians* he says 2 per cent. chloral hydrate is the best, and for *Oligochœta*, chloroform. For *Polyzoa* he uses menthol, overnight, then adds 40 per cent. formaldehyde.

C. F. **Rousselet Solution** (quoted from CARPENTER's "The Microscope") is 2 per cent. solution of hydrochlorate of cocaine, 3 parts; methylated spirit (*sic*), 1 part; water, 6 parts. This is the fluid used for Rotifera especially. Failing genuine cocaine, Peter Gray recommends stovaine.

DR. JOHN BAKER informs us that while Rousselet's fluid is good, in most laboratories it will be found convenient to substitute cocaine hydrochloride for the hydrochlorate and 90 per cent., alcohol for methylated spirit. John Baker's modified formula, which gives excellent results, is thus :—

Cocaine hydrochloride, 2 per cent. aqueous .	3 c.c.
Alcohol, 90 per cent.	1 c.c.
Distilled water	6 c.c.

For example, put Polyzoa in their natural water in a small watch-glass and remove the water until there is only just enough to cover them. Wait until they are well expanded. Add Rousselet's fluid, a little at a time. (Do not let drops of the fluid fall on the water, as the shock causes them to contract.) Continue until the watch-glass is nearly full, and then wait for ten minutes. Then add 10 drops (or less) of $\frac{1}{4}$ per cent. osmium tetroxide solution, and leave for three minutes. Wash repeatedly in changes of distilled water, and preserve in 4 per cent. formaldehyde.

14. Menthol. Now used with great success for anæsthetising large marine animals. Place latter in *clean* vessel, and sprinkle over surface of water, menthol crystals. As the latter dissolve the animals expand. In from twelve to twenty-four hours they may be transferred to a fixer. Very good for Anemones, Holothuria, Ascidia and many Mollusca. (Personal communication from Dr. E. J. ALLEN, Plymouth.)

15. Nicotin in solution (ANDRES, *Atti R. Acad. dei Lincei*, v, 1880, p. 9). Andres employs a solution of 1 grm. of nicotin in a litre of sea water. The animal is placed in a jar containing half a litre of sea water, and the solution of nicotin is gradually conducted into it by means of a thread, acting as a syphon, of such a thickness as to be capable of carrying over the whole of the solution of nicotin in twenty-four hours. See also *Mitth. Zool. Stat. Neapel*, Bd. ii, 1880, p. 123.

16. Chloroform may be employed either in the liquid state or in the state of vapour. The animals being extended, a watch-glass containing chloroform may be floated on the surface of the

water in which they are contained, and the whole covered with a bell-glass. As soon as they have become insensible they are killed by means of hot sublimate or chromic acid solution plentifully poured on to them. (KOROTNEFF, *Mitth. Zool. Stat. Neapel*, v, 1884, p. 233.)

Liquid chloroform is employed by squirting it in small quantities on to the surface of the water containing the animals. A syringe or pipette having a very small orifice, so as thoroughly to atomise the chloroform, should be employed. Small quantities only should be projected at a time, and the dose should be repeated every five minutes until the animals are anæsthetised.

Lee has seen large Medusæ very completely anæsthetised in extension in an hour or two by this method. ANDRES finds that it does not succeed with Actiniæ, as with them maceration of the tissues supervenes before anæsthesia is established.

PREYER (*Mitth. Zool. Stat. Neapel*, Bd. vii, 1886, p. 27) recommends chloroform water for star-fishes.

WADDINGTON employs a mixture of equal parts of 1 per cent. sol. of cocaine (or eucain) and saturated sol. of chloroform in water (sea or fresh), according to the habitat.

17. Ether and Alcohol may be administered in the same way. ANDRES has obtained good results with Actiniæ by the use of a mixture (invented by SALVATORE LO BIANCO) containing 20 parts of glycerin, 40 parts of 70 per cent. alcohol, and 40 parts of sea water. This mixture should be carefully poured on to the surface of the water containing the animals, and allowed to diffuse quietly through it. Several hours are sometimes necessary for this.

EISIG (*Fauna u. Flora Golf. Neapel*, xvi, 1887, p. 239) benumbs Capitellidæ by putting them into a mixture of 1 part of 70 per cent. alcohol with 9 parts of sea water.

OESTERGREN (*Zeit. wiss. Mik.*, xix, 1903, p. 300) makes a saturated (7 to 8 per cent.) solution of ether in sea or soft water and uses it either concentrated or diluted to about 1 per cent., and finds that it succeeds with all classes of aquatic animals.

CORI (*Zeit. wiss. Mik.*, vi, 1890, p. 438) recommends a mixture composed of 10 c.c. *methyl-alcohol* (of 96 per cent. strength), 90 c.c. water (fresh or sea water), and 0·6 grm. of sodium chloride (to be added only when fresh water is taken, the addition of the salt having for its object to prevent maceration). It may be well to add to this mixture a very few drops of chloroform (for *Cristatella ; Zeit. wiss. Zool.*, lv, 1893, p. 626).

18. Chloreton (Aceton Chloroform) is recommended for invertebrates and larvæ of *Rana* by RANDOLPH (*Zool. Anz.*, xxiii, 1900, p. 436). KRECKER (*Zeit. wiss. Zool.*, xcv, 1910, p. 383) takes solutions of $\frac{1}{3}$ to 1 per cent. for Oligochæta. SULIMA (*Zeit. Biol. Techn.*, Strasburg, i, 1909, p. 379) takes a mixture of 99 parts of

sea water and 1 of 10 per cent. sol. of chloreton in absolute alcohol, for *Scyllium* and *Anguilla*.

For Bryozoa, see Bessie Green, *Journ. Roy. Mic. Soc.*, 1914.

19. Hydrate of Chloral. Foettinger (*Arch. de Biol.*, vi, 1885, p. 115) operates by dropping crystals of chloral into the water containing the animals. For *Alcyonella* he takes 25 to 80 centigrammes of chloral for each 100 grm. of water. It takes about three-quarters of an hour to render a colony sufficiently insensible. He has obtained satisfactory results with marine and fresh-water Bryozoa, with Annelida, Mollusca, Nemertians, Actiniæ, and with *Asteracanthion*. He did not succeed with Hydroids.

Lo Bianco (*Mitth. Zool. Stat. Neapel,* Bd. ix, 1890, p. 442) employed for various marine animals freshly prepared solutions of chloral in sea water, of from $\frac{1}{10}$ to $\frac{1}{5}$ per cent. strength.

We have never had the slightest success with Nemertians.

Verworn (*Zeit. wiss. Zool.*, xlvi, 1887, p. 99) puts *Cristatella* for a few minutes into 10 per cent. solution of chloral, in which the animals sooner or later become extended.

Kükenthal (*Jena Zeit. Naturw.*, Bd. xx, 1887, p. 511) has obtained good results with some Annelids by means of a solution of 1 part of chloral in 1,000 parts of sea water.

The chloral method gives rise to maceration with some subjects, and has been said to distort nuclear figures.

20. Cocaine (Richards, *Zool. Anz.*, cxcvi, 1885, p. 3327). Richards puts a colony of Bryozoa into a watch-glass with 5 c.c. of water, and adds gradually 1 per cent. solution of hydrochlorate of cocaine in water. After five minutes the animals are somewhat numbed; $\frac{1}{2}$ c.c. of the solution is added, and ten minutes later the animals should be found to be dead in a state of extension.

This method is stated to succeed with Bryozoa, *Hydra*, and certain worms. It is the best method for Rotifers (Rousselet). It has also been recommended for *Aplysia*.

It has been pointed out (by Cori, in the paper quoted § 17) that, unfortunately, when fixing agents, such as sublimate solution, are added to the animals, the cocaine is thrown down on them as a white precipitate. This precipitate, however, may be redissolved afterwards in alcohol (Eisig).

Cocaine solutions cannot be depended on to keep for more than a few days.

21. Eucain. Harris (*Journ. Roy. Mic. Soc.*, 1900, p. 404) recommends a 1 per cent. solution of eucain hydrochloride, as giving far better results, with Vorticellidæ, Rotatoria, and Vermes. Rousselet (*ibid.*) reports favourably as to its action on Floscularíæ. It is stated to be perfectly stable in aqueous media. It dissolves in sea water to about 0·5 per cent.

22. **Hydroxylamin.** HOFER (*Zeit. wiss. Mik.*, vii, 1890, p. 318). Either the sulphate or, preferably, the hydrochlorate may be used. This should be dissolved in water (spring or sea water, according to the habitat) and exactly neutralised by addition of carbonate of soda. The organisms are placed in a solution diluted to about 0·1 per cent., for thirty minutes or less (as for Infusoria), to 0·25 per cent., for from fifteen minutes to one hour (*Hydra*), 1 per cent., one half to two hours (*Hirudo*) or as much as ten to twenty hours (*Helix* and *Anodonta*).

Hydroxylamin is a powerful reducing agent, and should therefore be well washed out before treating with easily reducible fixing agents.

23. **Chloride or Sulphate of Magnesium.** TULLBERG (*Arch. Zool. Expér. et Gen.*, x, 1892, p. 11). For Actiniæ, a 33 per cent. solution of the chloride should be very slowly added to the water containing the expanded animal, until the vessel contains 1 per cent. of the salt (thus for 1 litre of sea water 33 c.c. of the solution must be added). The addition must be completed within half an hour, and thirty minutes later the animal may be fixed.

For terrestrial and fresh-water Invertebrates rather stronger solutions should be used.

REDENBAUGH (*Amer. Natural.*, xxix, 1895, p. 399) takes the sulphate either added in crystals to the sea water containing the animals until a saturated solution is obtained, or in the shape of a saturated solution into which they are thrown (Annelids).

See also MAYER, *Biol. Bull. Wood's Hole*, xvii, 1909, p. 341 (puts direct into sol. of 70 per cent. strength).

24. **Poisoning** by small doses of some fixing agent is sometimes good. LO BIANCO kills *Ascidia* and *Rhopalœa* in an extended state (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 471) by pouring a little 1 per cent. chromic acid on to the surface of the water containing them, and allowing it to diffuse slowly into it. About twelve to twenty-four hours is necessary. He kills *Ciona* in a similar way with a mixture of 1 part of 1 per cent. chromic acid and 9 parts of 49 per cent. acetic acid.

Osmic acid, or Kleinenberg's solution, is sometimes employed in the same way.

Lee has seen Medusæ killed in a satisfactory manner by means of crystals of corrosive sublimate added to the water containing them.

Morphia, Curare, Strychnin, Prussic Acid, and other paralysing drugs, have also been employed.

25. **Asphyxiation** may be sometimes successfully practised. Terrestrial Gastropods may be killed for dissection by putting them into a jar quite full of water that has been deprived of its air by boiling, and hermetically closing it. After from twelve to twenty-four hours they are generally found dead and extended. The effect is obtained somewhat quicker if a little *tobacco* be added to the water.

Good results are sometimes obtained with aquatic animals by simply leaving them to exhaust the oxygen of the water in which they are contained. We have sometimes succeeded with Holo-

thuriæ and other Echinoderms in this way. WARD (see *Amer. Nat.*, xxv, 1891, p. 398) has succeeded with Hydroids, Actiniæ, and similar forms, and UEXKÜLL (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 463) with Echinids.

Marine animals are sometimes successfully killed by simply putting them into *spring water.*

Warm Water will sometimes serve to immobilise and even kill both marine and fresh-water organisms.

Carbonic Acid Gas has been recommended (by FOL, *Zool. Anz.*, cxxviii, 1885, p. 698). The water containing the animals should be saturated with the gas. The method is stated to succeed with most Cœlenterata and Echinodermata, but not with Molluscs or Fishes. We have had most excellent results with small Annelids and Hirudinea. It is not necessary to employ a generator for obtaining the gas. It suffices to take an ordinary " soda-water " syphon, and squirt its contents into the water containing the animals.

Narcotisation is very rapidly obtained with very small animals, but much more slowly with larger ones. For instance, *Stylaria proboscidea*, we find, is paralysed in a few *seconds ;* a small *Nephelis* of 15 or 20 mm. in length, will require about five *minutes ;* and a large *Nephelis*, of from 10 to 15 cm., will require as many *hours.*

UEXKÜLL (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 463) has paralysed Echinids very rapidly with carbonic acid, likewise a small Teleostean fish ; whilst *Scyllium* and Crustaceans were affected much more slowly, and mussels not at all.

26. Peroxide of Hydrogen. VOLK (*Zool. Anz.*, xix, 1896, p. 294) kills Rotatoria by means of one or two drops of a 3 per cent. solution added to 1 c.c. of the water containing them.

27. Anæsthesia of Animals for Cell Studies. J. McA. KATER (*Science*, vol. 82, 1935) finds sodium amytal satisfactory for rabbit liver, there being no difference between control and anæsthetised animals after half an hour. The dose was 3 grains for an animal of 5 to 6 lbs. weight. HIRSCH (*Z. Zellf.*, 1932) and DUTHIE (*Proc. Roy. Soc. B.*, 1933) used Cibalgin for mice, 0·6 c.c. of $\frac{1}{10}$ solution.

CHAPTER III

FIXING AND HARDENING

28. The Functions of Fixing Agents. The meaning of the term "fixing" has been explained above (§ 2). Here is an example showing the necessity of fixation.

If a portion of living retina be placed in aqueous humour, serum, or other so-called "indifferent" medium, or in any of the media used for permanent preservation, it will be found that the rods and cones will not preserve the appearance they have during life for more than a very short time; after a few minutes a series of changes begins to take place, by which the outer segments of both rods and cones become split into discs, and finally disintegrate so as to be altogether unrecognisable, even if not totally destroyed. Further, in an equally short time the nerve-fibres become varicose, and appear to be thickly studded with spindle-shaped knots; and other post-mortem changes rapidly occur. If, however, a fresh piece of retina be treated with a strong solution of osmic acid, the whole of the rods and cones will be found perfectly preserved after twenty-four hours' time, and the nerve-fibres will be found not to be varicose. After this preliminary hardening, portions of the retina may be treated with water (which would be ruinous to the structures of a fresh retina), they may even remain in water for days without harm; they may be stained, acidified, hardened, imbedded, cut into sections, and mounted in either aqueous or resinous media without suffering.

This example shows that one of the objects aimed at in fixing is to impart to tissues the degree of *hardening* necessary to enable them to offer such mechanical resistance to *post-mortem* change and to the processes of after-treatment as not to suffer change of form. Another important function of fixing is to *render insoluble* elements of cells and tissues that would otherwise be more or less dissolved out by the liquids employed in the after-treatment. A third and highly important function of fixing agents consists in producing *optical differentiation* in structures. By coagulating the elements of tissues and cells, fixing agents *alter their indices of refraction,* raising them in varying degrees. They do not act in an equal degree on all the constituent elements of cells and tissues, but raise the index of some more than that of others, thus producing optical differentiation where there was little or none before.

Compare the aspect of the epithelium of the tail of a living tadpole, observed in water, with its aspect after the action of a little diluted solution of Flemming. In the living state the protoplasm of its cells has a refractive index little superior to that of water, and consequently so low an index of visibility that hardly any structure can be made out in the object. But as soon as the protoplasm has been sufficiently

coagulated by the reagent the refractive indices of some of its elements will have been raised to above that of balsam, the chromatin of the nuclei will be brought out, and other structures be revealed where none was visible before.

29. The Action of Fixing Agents consists in *coagulating* and rendering insoluble certain of the constituents of tissues.

According to FISCHER (*Fixirung, Färbung, und Bau des Protoplasmas*, Jena, G. Fischer, 1899), the coagulation which constitutes fixation is, in the case of the liquid and semi-liquid constituents of tissues, *always* a phenomenon of *precipitation*. The more solid constituents (such as fibrils that are visible during life, nucleoli, and the like) he admits may be acted on by fixing reagents without the formation of any visible precipitates. But all the liquid ones, in so far as they are fixed at all, are visibly precipitated in special *precipitation forms*, which vary according to the precipitant. Each fixing agent gives its own characteristic *fixation image*, which may be more or less lifelike, but can never be absolutely so. Fischer gives copious descriptions of the precipitation forms of the chief organic compounds found in tissues, and of the precipitation powers of the chief fixing agents, which the reader will do well to study.

It seems to be a consequence of Fischer's theory of fixation by precipitation that the most energetic fixing agents should always be found amongst the most energetic precipitants. But on the showing of his experiments this is not so. For instance, it is allowed on all hands that osmic acid is a most energetic fixative. But Fischer finds (*op. cit.*, pp. 12—14, 27) that it is a very incomplete and weak precipitant. Or, to take a contrary instance, he finds that picric acid is an energetic precipitant of the majority of cell constituents ; but surely every cytologist must admit that it is not a highly energetic fixative !

It would seem to follow, from these instances and from other similar ones, that Fischer's tables of precipitating power cannot be taken as a measure of the fixing power of the reagents. And further, the study of the fixation images of tissues afforded by osmic acid, formaldehyde, and other reagents, seems to show that the coagulation brought about by them is in part accompanied by the formation of visible precipitates, but in part not so, and that they may do their work to a larger extent than he seems to admit through a *homogeneous coagulation*. Fischer, studying the effects of certain fixatives on albumose, states that mixing 10 per cent. slightly acid deutero-albumose with Altmann's bichromate-osmic fluid causes a precipitate of granules of from 1 to 3 μ in diameter, while corrosive sublimate of 7 per cent. causes granules of 0·4 to 1 μ in size ; one might be led away, as was Fischer, to consider that Altmann's fluid used on cells therefore causes artifacts to appear. As a matter of fact corrosive

sublimate is much more dangerous than Altmann's fluid, in this respect, while Altmann's fluid merely preserves cell granules which are visible *intra vitam*. The ground protoplasm after corrosive is more granular and coarsely reticulate than after Altmann; this does not apply to mitochondria or Golgi elements which are often very badly preserved with corrosive.

Fischer (*op. cit.*) says, "Many kinds of cell contents, indeed the majority, have an alkaline reaction, and are thereby quite inaccessible to the precipitating action of certain agents, such as osmic acid, or bichromate; and the action of certain other fixatives, such as platinum chloride or chromic acid, is more or less hindered by the presence of free alkalies. For neither the chromic acid (of the Flemming), nor the platinum chloride (of the Hermann) would be adequate to act as acidifiers to the osmic acid of the mixtures."

Our experience is directly contrary to these conclusions of Fischer: it is common knowledge among modern workers that a cell fixed in acetic acid-containing solutions has a more "raked out" appearance than when the acetic acid is omitted: this applies not only to cell granules, but to the appearance of the ground cytoplasm, nucleoli, and chromatin filaments. *The statement that osmic acid must be acidified before it will fix all parts of the cell is also contrary to general experience.* Formalin neutralised gives a gentler and more precise fixation than acid formalin. While Fischer's results may be excellent so far as they concern his theoretical conclusions on the fixation of weak solutions of egg-white, etc., too much attention should not be paid to one who is not thoroughly acquainted with practical cytology and histology.

It has been pointed out by some workers (*e.g.* UNNA, *Arch. f. mikr. Anat.*, lxxviii), that many of the fixing reagents come under the category of oxidisers; Unna places great importance on the fact that some of the most successful fixers are oxidisers, *e.g.* OsO_4,$K_2Cr_2O_7$, CrO_3; but formalin, admittedly a splendid reagent, is a reducer while picric acid and corrosive sublimate are feeble oxidisers, and that only under special conditions.

Of the ten common reagents used for fixing, only four are marked oxidisers, and Unna's generalities with reference to the significance of oxidisers may not be the correct explanation. Generalities such as made by Unna with reference to the *rôle* of oxidisers in fixation resemble like claims which have been made with reference to the supposed necessity for the constant use of an acid in fixatives (and preferably acetic acid which may be a dangerous reagent).

With regard to the relative values of oxidisers or reducers in fixing cytoplasm or nucleus, formalin (reducer) * and OsO_4

* See, however, *Blum*, Enzykl. d. mikr. Tech., 1910.

(oxidiser) are both famous cytoplasm fixers, while acetic acid (neither oxidiser nor reducer), or alcohol (reducer) and CrO_3 (oxidiser) are well-known nuclear fixatives.

Helly's fluid, formol-bichromate or formol-Flemming are all splendid fixatives, and mixtures of both oxidisers and reducers; it is difficult to see how Unna's theories can apply here. In the case of Flemming fluid, without acetic acid, it is certain that the fixation process in so far as it concerns the OsO_4 in this mixture, is not solely an oxidising process, at least of the same nature as the fixation reaction by the chromic acid (CrO_3). In a word, fixation of the cell by various kinds of chemical reagents is an extremely complicated matter concerning a large number of organic substances whose reactions to the chemical used are probably different in most cases.

30. The Characters of the Usual Fixing Agents. These agents are as follows :—

A.
1. Osmium tetroxide,
2. Formaldehyde gas,
3. Chromium trioxide,
4. Bichromate of potassium,
5. Platinum chloride,
6. Mercury bichloride, all in water.

B.
7. Picric acid in water,
8. Alcohol,
9. Nitric acid and
10. Acetic acid in water.

Chloroform and urea are also used. In the group marked A are arranged the more valuable reagents, in B the less valuable or destructive ones. Good fixatives can be made from the substances in group A without using any of the reagents in group B. The latter contain most of the reagents useful for chromosome work, the former, reagents useful for fixing the cytoplasm and "resting" nucleus.

From group A have been made the following mixtures: Altmann, Champy, and Flemming and Hermann-without-acetic acid; these are among the best mixtures known. Then there are formol (5 per cent. to 10 per cent.), Regaud, Helly, formol-Müller and formol-Flemming, which are so good for mammals. Good general microanatomical fixatives from both groups A and B are Zenker, Bouin, Gilson-Petrunkewitsch and corrosive acetic, but these all destroy much of the cell-contents, and give an incorrect picture of the cell, excepting of chromatinic structures, for which they are indicated.

A good fixing agent should first of all *preserve* all the elements it is desired to fix. But that is not enough; it should also give good optical *differentiation.* and should have sufficient power of

penetration to ensure that small pieces of tissue be equally fixed by it throughout. No single substance or chemical compound fulfils all that is required of a good fixing agent; hence it is that all the best fixing agents are *mixtures*. Osmic acid, for instance, fulfils some of these conditions, but not all of them. It kills rapidly and preserves admirably the elements of cytoplasm, but nuclei not so well. But the optical differentiation that it gives, though sometimes good, is often very inferior. For osmic acid, by coagulating in nearly equal degrees alike the spongioplasm (the plastin reticulum) and the hyaloplasm (the enchylema) of the cell-body, and the chromatin of nuclei, raises alike the refractive indices of all of them; so that if the fixing action have been in the least degree overdone, the cells acquire a homogeneous aspect in which the finer details are obscured by the general refractivity of the whole. If now, instead of using it pure, it be used in combination with chromic acid, a better differentiation is obtained; for chromic acid, whilst enhancing, and at all events not interfering with the fixation of chromatin, serves to facilitate penetration and to counteract the excessive action of the osmic acid on the protoplasm, so that the cells come out less homogeneous and with more detail observable in them.

Descriptive embryologists often use strange illogical mixtures containing both reducible substances and violent reducers, both fat-solvents and fat-preservers, mixed together without regard for the chemistry of fixation. It is only the logically planned fixative that is found generally useful, and which stands the test of time. Fixation falls under three broad headings :—

1. *Micro-anatomical*, in which correct preservation of cell aggregates, without shrinkage or expanding, is the *desideratum*. Such is the aim of most descriptive embryologists.

2. *Cytological* from the point of view of *the chromosome* or *nucleus*.

3. *Cytological* from the point of view of fixing the cell in a state which *most resembles its condition when alive*; also so as to identify the cell elements, especially in the cytoplasm.

In most cases the results attained by workers belonging to sections 1 and 2 can truly be said to give a caricature of the cell *intra vitam*. We give below a general classification of fixatives, those in (*a*) being fixatives causing the maximum disturbance and destruction in the individual cell, those in (*c*) the least.

A great deal, however, depends on the accessibility of the cells to the fixative, and as to whether vertebrate or invertebrate material is being used.

(*a*) Carnoy, Petrunkewitsch, alcohol, Gilson, picro-nitric, etc. Fat, mitochondria, Golgi apparatus, and often delicate yolk discs do not show after these. (Using alcohols and xylol subsequently.)

(*b*) Bouin, Zenker, corrosive acetic, Flemming-with-acetic acid, etc.

Mitochondria and Golgi apparatus rarely show after these, except possibly in mammals, where these cell inclusions are more resistant than in invertebrata. Fats show with the last-mentioned fixative.

(*c*) Osmic acid, Flemming-without-acetic, Champy, Altmann, formalin, Mann's mercury-osmic liquid, Sjövall's method, etc. Preserve all formed granules. (Using fluids subsequently as above.)

In section (*c*) the formol alone will not preserve fat; but see Sjövall's method (§ 718).

The fixatives have not been classed according to how they themselves alone affect the contents of the cell, but according to how they preserve the cell preparatory to its treatment in the liquids necessary for imbedding and sectioning.

Injurious liquids which should never be used in cytological fixation (3, *vide supra*) are acetic acid, chloroform and alcohol. Acetic acid is probably the most destructive to delicate lipins, and its use, except where chromosomes are being studied, is rarely indicated; any worker who uses acetic acid in his fixing mixtures cannot hope to get a correct picture of any part of his cell, possibly excepting the chromosomes (not the resting nucleus). The most valuable fixatives are osmium-tetroxide, bichromate of potassium, chromium-trioxide, and formaldehyde, possibly in the order named; the most valuable mixtures are Müller-formol (or Helly), Flemming-without-acetic, Altmann, and Champy; the three latter approach as near perfection as present-day technique allows. Altmann's fluid ($K_2Cr_2O_7 + OsO_4$) we find to be a splendid mixture. In no case, except in small invertebrates, do these fixatives (excluding formol) give a true fixation of cell aggregates; this is due to their inferior penetrating powers, and to an unevenness of penetration. Small invertebrates, both marine and fresh-water, and small pieces of tissues, are usually exquisitely preserved in chrome-osmium mixtures, but are not then generally suitable for staining and mounting whole, especially for staining in carmine mixtures.

31. Penetration of Fixatives. We cannot enter more fully into the various results got by mixing globulin, albumin, etc., with fixing mixtures. These experiments are reviewed in an interesting manner by John Baker (*Cytological Technique*, 1933). But more recently J. Z. Young (*Nature*, May 18th, 1935) has drawn attention to the great improvement got in penetration of such weakly penetrating mixtures as Champy and Flemming, by the addition of 0·9 or 0·75 per cent. NaCl. In the Dublin laboratory, William Boyle and the writer have used such "isotonic" fixatives, and in some cases the improvement in penetration has been astonishing.

In such material as mammalian testis or molluscan ovotestis, it is certain that more tubules are properly fixed than in any preparations hitherto made over a period of many years by the writer. It is remarkable that HIRSCH and JACOBS (*Zeit. f. Zellf.*, 1926) came to the conclusion that in just such mixtures as were used by Young, Boyle and the writer, the addition of salt was of no value. Hirsch and Jacobs used crayfish mesenteron in which the difficulty of penetration is obviously not so great as in liver or testis, for example. We agree with Hirsch and Jacobs that cells on the outside of a tissue are not visibly better fixed by fixatives containing NaCl, but this is not true of the cells or tubules more deeply situated. Therefore we advocate trying the addition of 0·75 to 0·9 per cent. NaCl to chrome-osmium, chrome-formalin and formalin mixtures. There is, however, one point that should be mentioned. Boyle has found that Flemming's and Champy's fluids disintegrate in a few weeks when salt is added. Under the separate paragraphs giving formulæ this matter has been further treated.

Since the addition of NaCl to fixatives of the chrome-osmium formalin types improves the penetration, it would seem unfruitful to review some of the recent work on penetration of single substances in water which has been done without the addition of NaCl. Refer, for instance, to the interesting and valuable paper by Miss B. M. L. UNDERHILL (*J. Roy. Micr. Soc.*, lii, 1932).

32. Period of Fixation. The beginner is often puzzled by the varying directions given as to length of time the fixative is to be used. For example, the variations of Flemming's fluid are used sometimes for one day up to as long as a week. The late Professor Doncaster always used Flemming acetic for one hour only. The explanation is, of course, that if you merely want chromosomes, the chromic and osmic will fix proteids in a short time—while the worker who wants to study the fats * must needs prolong the immersion until all these subtle bodies are sufficiently hardened to resist the solvent effects of alcohol and xylol or toluol.

In the same way there are many failures in the Regaud method, because the proper times are not adhered to strictly. Most of the tribe of corrosive acetic alcohol fixatives do quite well when used for an hour or so; it is conventional to use most ordinary fixatives overnight. Some well-known mixtures, such as Da Fano's formol-cobalt and Aoyama, are really good preservatives, and we constantly use them to store material. The beginner must read the directions for time of fixation given under each formula.

For routine zoological work Bouin's picro-formalin-acetic is recommended. Gilson-Petrunkewitsch is a fixative which is easy to work and generally better than corrosive sublimate acetic.

* But see § 734.

For **routine vertebrate histological work** Zenker, Susa and Helly's Zenker-formol are indicated.

We think *the beginner* should avoid such things as liquid of Flemming and similar mixtures.

Picric acid gives a fair though weak fixation, with very good penetration, is easy to manage, and does not make tissues brittle, which sublimate easily may do. Pure diluted formol is not bad, and very easy to manage.

Speaking generally, osmic acid, chromic acid, bichromates, chloride of platinum, and the majority of the compounds of the heavy metals, are *hindrances* to staining; whilst heat, alcohol, trichloracetic acid, formol, corrosive sublimate, picric acid, and acetic acid, are *neutral*, or even *favourable*, in this respect.

33. Validity of Fixation Image. In the problem of fixation it is necessary to notice that two criteria may be taken, firstly, the condition of the ground cytoplasm and nucleus; secondly, the condition of the various categories of formed bodies, such as mitochondria fat, Golgi apparatus, paraglycogen and so on. The condition of the ground cytoplasm is usually of little interest to those working in the formed bodies in the protoplasm, though it is generally recognised that only the smooth appearance got by osmium and chrome-osmium fixation resembles the condition *intra vitam*. Thus the work of Fischer and Hardy is of little importance to those working on the cytoplasmic inclusions, because the cytoplasmic inclusions are not usually properly demonstrated by fixatives which cause intense coagulation and production of artifacts in the ground cytoplasm. The condition of the ground cytoplasm may, however, be quite different with two really good methods for demonstrating the cytoplasmic inclusions, for example, with the Weigl and the Nassanow methods, the former containing corrosive sublimate, the latter chrome salts. That the methods used to demonstrate the cytoplasmic inclusions do not merely produce artifacts as claimed by Walker (*Proc. Roy. Soc.* B., 1927) can be shown by watching the fixation of cells under the microscope, and by comparing the finished preparations with the living cells. This may be done easily in tissue cultures, or with smears of insects or molluscan gonads. It must be admitted, however, that in the finished slides such fixatives as Bouin's fluid (§ 115), more so alcoholic Bouin or acetic corrosive sublimate (§ 68), produce preparations of cytoplasm which show little resemblance to the cell or organism in the living condition. It is admitted widely nowadays that osmic and chrome-osmic fixatives are the best cytoplasm fixatives known to us.

In the case of chromatin it may be pointed out that, in the living state, the intact nucleus is optically empty, except for the nucleolus, and that the structures which we see after fixation may

be artifacts. In the past, to these objections, the answer has been made that with a wide variety of agents we obtain essentially the same fixation image and this constancy must rest on a physical basis, whether or not there is an optical differentiation in the living tissue. To this may now be added some observations of the late Dr. Belâr. He showed that in the spermatocytes of the grasshopper, *Stenobothrus*, the chromosomes of the living cells are visible at all stages of meiosis, while in another species, *Dissoteira*, the nucleus appears empty and the chromosomes are first seen after fixation (*Protoplasma*, ix, 1930, p. 209). In the paper cited, and in many others, Belâr gives photomicrographs of the same cells, in the living state, with the chromosomes clearly visible, after fixation, when the chromosomes may still be seen clearly, and after fixation and staining. A comparison of these figures will convince anyone that after fixation and staining the condensed chromosomes have essentially the same form as in the living state. It seems safe to say, therefore, that as far as such gross morphological details are concerned, our nuclear fixatives may be relied on to give valid images of the living chromosomes. It should be pointed out, however, that for the finer details of nuclear organisation, for which the living material gives us little evidence, we cannot be so certain of our grounds.

From the practical standpoint, checking fixation images in living material is often difficult, and the method is limited in its application, and it may be asked by what criterion may we judge the quality of fixation. Unfortunately, there is no objective standard recognised and the matter becomes subjective. The careful investigator will try out a number of fixatives on new material, in order to eliminate any features which result from the use of a single reagent.

34. The Practice of Fixation. *See that the structures are perfectly living at the instant of fixation, otherwise you will only fix pathological states or post-mortem states.*

Some observers have made special observations on the effect of delay in fixation; J. Thornton Carter (*Phil. Trans. Roy. Soc.*, Series B, vol. ccviii, 1917) has made some interesting experiments on the finely granular ameloblasts in the developing teeth of the pike. He noticed that the cytoplasm gave evidence of marked changes unless fixed within three minutes of "death"; these changes were manifested by the behaviour of the cytoplasmic granules to stains; the selectivity of the latter was progressively altered as the rapid post-mortem changes were set in action.

Fixation is generally performed by *immersion* of the objects in the fixing liquid. In this case, everything should be done to facilitate the *rapid penetration* of the fixing agent. To this end let the structures be divided into the smallest portions that can conveniently be employed, and if entire organs or organisms are

to be fixed whole, let openings, as large as possible, be first made in them.

The penetration of reagents is greatly facilitated by *heat*. You may warm the reagent and put it with the object to be fixed in the paraffin stove, or you may even employ a fixing agent heated to boiling-point (as boiling sublimate solution for certain corals and Hydroids, or boiling absolute alcohol for certain Arthropods with very resistant integuments). But this should only be done as a last resource.

On the other hand, very cold reagents are nowadays used for some purposes.

Let the *quantity* of fixing agent employed be *many times* the volume of the objects to be fixed. If this precaution be not observed the composition of the fixing liquid may be seriously altered by admixture of the liquids or of the soluble substances of the tissues thrown into it. For a weak and slowly acting fixing agent, such as picric acid, the quantity of liquid employed should be in volume about one hundred times that of the object to be fixed. Reagents that act very energetically, such as Flemming's solution, may be employed in smaller proportions.

But fixation may also be performed by *injection* of the fixing liquid into the objects, thus ensuring a more rapid and thorough penetration of *voluminous* objects. See for this practice the methods of fixation by injection of GOLGI, DE QUERVAIN, MANN, and others, given under *Nervous System*.

BRAUS and DRUENER (*Jena Zeit. Naturw.*, Bd. xxix, 1895, p. 435) fix fishes *by injection* through the *bulbus aortæ*. The vessels are first washed out with normal salt solution, and the fixing liquid is then thrown in.

KOLMER (*Anat. Anz.*, xlii, 1912, p. 47) fixes thus even large mammals (Chimpanzee, Goat). He first washes out with RINGER's solution.

35. Washing Out. Careful *washing out* (by which is meant the removal from the tissues of the excess of uncombined fixative) is necessary in order to get tissues to stain properly. But it is not always equally imperative. Alcohol and formaldehyde do not require washing out before staining; acetic and picric acid only for some stains; sublimate will allow of staining even if not washed out, but allows of a sharper stain if well washed out; all osmic, chromic, and platinic liquids require very thorough washing out.

It is important to use the *appropriate liquid for washing out* the fixing agent after fixation. It is frequently by no means a matter of indifference whether water or alcohol be employed for washing out. Sometimes water will undo the whole work of fixation (as with picric acid). Sometimes alcohol causes precipitates that may ruin the preparations. Objects fixed in alcohol, formol, acetic acid, picric acid, or nitric acid require to be washed out with alcohol, or at least with some hardening

liquid, whilst those that have been fixed with osmic or chromic acid, or with one of the other compounds of the heavy metals, require *in general* to be washed out with water. Sublimate, however, is best washed out with alcohol.

Use *liberal quantities* of liquid for washing.

For almost any objects which are to be washed out in water, it is convenient to use wide-mouthed vessels containing the animals or pieces of tissue. Gauze, or mosquito netting of a mesh smaller than the objects, is tied over the mouth and the vessel is placed under a running tap.

Very minute objects may be tied in a special phial and placed in a larger vessel, or alternatively distilled water may be pipetted on them at intervals.

The process of washing out is greatly facilitated by *heat*. Picric acid, for instance, is nearly twice as soluble in alcohol warmed to 40° C. as in alcohol at the normal temperature (Fol.).

The correct period for washing out Golgi apparatus and mitochondrial material is extremely important (see § 688).

36. Fixation of Marine Animals.* The tissues of *marine organisms* are as a general rule more refractory to the action of reagents than those of corresponding fresh-water or terrestrial forms, and fixing solutions should in consequence be stronger (about two to three times). It is generally recommended when possible to make up such fixing solutions, using sea water instead of *aqua pura*.

Marine animals ought to be *freed from the sea water* adherent to their surface before treating them either with alcohol or any fixing reagent that precipitates the salts of sea water. If this be not done, the precipitated salts will form on the surfaces of the organisms a crust that prevents the penetration of reagents to the interior. Fixing solutions for marine organisms should therefore be such as serve to keep in a state of solution, and finally remove, the salts in question. If alcohol be employed, it should be *acidified* with hydrochloric or some other appropriate acid. Picro-nitric acid is a fixing reagent that fulfils the conditions here mentioned. (On this subject see Mayer, in *Mitth. Zool. Stat. Neapel*, ii (1881), pp. 1 *et seq.*, and Allen and Browne in "Science of the Sea," John Murray, 1912).

37. Hardening. The process of hardening is distinguished from that of fixing as being directed to the attainment of a degree of consistency sufficient to allow of soft tissues being cut into sections without imbedding. It is an *after-process*, and only ranks as a *special* method.

Methods of imbedding have now been brought to such a degree of perfection that the thorough hardening of soft tissues that was formerly necessary in order to cut thin sections from them is, in the majority of cases, no longer necessary. But there are some exceptions. Such are,

* Not yet properly studied. The subject is bound up with the matters raised in § 31.

for instance, the cases in which it is desired to cut very large sections, such as sections of the entire human brain.

The reagents employed for hardening are for the most part of the same nature as those employed for fixing. But it does not follow that all fixing agents can be employed for hardening. Corrosive sublimate, for instance, would be most inappropriate as a hardening agent.

The Practice of Hardening. Employ *in general a relatively large volume* of hardening liquid, and change it very frequently. If the volume of liquid be insufficient, its composition will soon become seriously altered by the diffusion into it of the soluble substances of the tissues ; and the result may be a macerating instead of a hardening liquid.

Hardening had better be done in tall cylindrical vessels, the objects being suspended by a thread, or muslin bag, or otherwise, at the top of the liquid. This has the advantage of allowing diffusion to take place as freely as possible, whilst any precipitates that may form fall harmlessly to the bottom ; or, they may be laid on a layer of cotton-wool, or filter-paper, or spun glass.

In general, *begin* hardening with a *weak reagent,* increasing the strength gradually, as fast as the tissues acquire a consistency that enables them to support a more energetic action of the reagent.

Let the objects be removed from the hardening fluid as soon as they have acquired the desired consistency.

38. With regard to **Shrinkage** it has been known for years that the critical period was mainly at dehydration and dealcoholisation. Whence the use of such oils as cedar wood, followed by a washing out in benzol or xylol. The introduction of fluids for imbedding, which obviate the use of the xylol and ethyl alcohol, or carbon bisulphide-alcohol stages, has done much to get over this shrinkage (§ 125). For many years it has been usual to double-imbed in celloidin and wax. This takes longer, but it does get rid of a great part of the shrinkage.

39. Fixation by Altmann's Freezing-Drying Method. Altmann froze organs, or blocks of tissue, and dehydrated them at low temperatures (— 15° C. to — 20° C.) in a vacuum, and showed that such material was preserved without shrinkage and was suitable for the localisation of substances normally within the body (Altmann, *Die Elementarorganismen und ihre Beziehungen zur den Zellen,* 1890, Leipzig: Veit). This method has received scant attention until recently when Gersh (*Anat. Rec.*, liii, 1932, p. 309) greatly improved the apparatus required for the successful application of this method. Briefly stated, the method, as developed by Gersh, consists in freezing tissue in liquid air (by simple immersion) and then dehydrating at about — 20° C. in a vacuum. When completely dried the tissue is placed in melted paraffin and after infiltration is imbedded. It may now be sectioned and treated as desired. This method is finding wide application, by Bensley and Gersh and their students, in the study of cell structures which have been unaltered by the chemical and physical reagents commonly employed in microscopic technique.

CHAPTER IV

FIXING AND HARDENING AGENTS—MINERAL ACIDS AND THEIR SALTS

40. Osmic Acid. The tetroxide of osmium (OsO_4) is the substance commonly known as osmic acid, though it does not possess acid properties. It is extremely volatile, and in the form of an aqueous solution becomes partially reduced with great readiness in the presence of the slightest contaminating particle of organic matter. It is generally believed that the aqueous solutions are reduced by light alone, but this is not the case: they may be exposed to the light with impunity *if dust be absolutely denied access to them.*

The solution of osmic acid in chromic acid solution is not, like the solution in pure water, easily reducible, but may be kept without any special precautions. BOLLES LEE used to keep the bulk of osmium in the shape of a 2 per cent. solution of osmic acid in 1 per cent. aqueous chromic acid solution. This solution served for fixation by osmium vapours, and for making up solution of Flemming, which is the form in which osmium is most generally employed. A small quantity of osmic acid may also be made up in 1 per cent. solution in distilled water, and kept in a drop-bottle with grooved stopper, from which quantities can be obtained when required without removing the stopper.

CORI (*Zeit. wiss. Mik.*, vi, 1890, p. 442) finds that solutions in distilled water keep perfectly if there be added to them enough permanganate of potassium to give a very slight rosy tint to the liquid. From time to time, as the solution becomes colourless, further small quantities of the salt should be added, so as to keep up the rosy tint.

BUSCH finds that the addition of sodium iodate hinders reduction (*Neurol. Centralb.*, xvii, 1898, p. 476).

PINTNER finds that a slight addition of corrosive sublimate has the same effect, *e.g.* 10 drops of 5 per cent. solution of sublimate added to 100 c.c. of 1 per cent. solution of osmic acid.

For the Kopsch, Mann-Kopsch and Sjövall methods the osmic acid solution *should be free* from all traces of chrome and platinum salts, etc. We recommend that osmic acid be bought in ½ grm. tubes and a fresh supply be made up at frequent intervals.

For the so-called "regeneration" of reduced solutions, see *previous editions.*

Osmic acid is met with in commerce in the solid form in sealed tubes. The assigned weights should be checked, as they may vary greatly.

Fixation by the Vapours. This is indicated in most of the cases in which it is possible to expose the tissues directly to the

action of the vapour. The tissues are treated as described in § 717. Very small objects, such as isolated cells, are simply placed on a slide, which is inverted over the mouth of the bottle.

They remain there until they begin to turn brown (isolated cells will generally be found to be sufficiently fixed in thirty seconds: whilst in order to fix the deeper layers of relatively thick objects, such as retina, an exposure of several hours may be desirable). It is well to wash the objects with water before staining, but a very slight washing will suffice. For staining, methyl-green may be recommended for objects destined for study in an aqueous medium, and, for permanent preparations, alum-carmine, picro-carmine, or hæmatoxylin.

In researches on nuclei, it may be useful to employ the vapours of a freshly prepared mixture of osmic and formic or acetic acid (Gilson, *La Cellule*, i, 1885, p. 96).

The reasons for preferring fixation by the vapour are that osmic acid is more highly penetrating in vapour than in solution; that the arduous washing out required by the solutions is done away with; and that all possibility of deformation through osmosis is eliminated. See also under "Cramer's Method," § 717.

Fixation by Solutions. Osmic acid is now very seldom used *pure* in the shape of solutions. When, however, it is so employed it is used in strengths varying from $\frac{1}{20}$ to 2 per cent. *Never more than* 2 per cent. need be used.

On account of its feeble penetrating power the objects to be fixed should be as small as possible.

The solutions should be kept protected from bright light during the immersion of tissues. (This precaution is not necessary if Flemming's or Hermann's solution be used.) If the immersion is to be a long one the tissues must be placed with the solution in well-closed glass-stoppered vessels. The objects may be deemed to be fixed as soon as they have become brown throughout. But see "Mann-Kopsch Methods," § 710.

After-Treatment. The excess of osmic acid must be well washed out before proceeding to any further steps in preparation; water should be used for washing. Notwithstanding the greatest care in soaking, it frequently happens that some of the acid remains in the tissues, and causes them to *blacken* in time, and in any case hinders staining. To obviate this blackening it has been advised to soak them for twenty-four hours in a solution of bichromate of potash (Müller's solution or Erlicki's will do), or in 0·5 per cent. solution of chromic acid, or in Merkel's solution. The treatment with bichromate solutions has the great advantage of highly facilitating staining with carmine or hæmatoxylin. Max Schultze recommended washing, and mounting permanently in acetate of potash; Fol, treatment with a weak solution of carbonate of ammonia. But the best plan of all is to properly *bleach* the preparations. See "Bleaching." This may be done by means of *peroxide of hydrogen*. Overton (*Zeit. wiss. Mik.*, vii, 1890, p. 10) finds that it is completed in a few minutes in a

mixture of 1 part commercial peroxide with 10 to 25 parts 70 per cent. alcohol. The commercial peroxide, slightly aciduated with HCl, will keep well in the dark; but the mixture with alcohol must be made fresh for use. According to BRISTOL (*Amer. Natural.*,xxvii, 1893, p. 176) the peroxide acts best in the sun. BINET (*Journ. de l'Anat. et de la Physiol.*, xxx, 1894, p. 449) has successfully used permanganate of potash. MANN (*Methods*, etc., p. 83) takes a solution of 0·25 per cent., and treats the browned tissues with 1 part of saturated solution of sulphurous acid to 9 of normal salt solution. . . . MÖNCKEBERG and BETHE (*Arch. Mik. Anat.*, liv, 1899, p. 135) have succeeded in satisfactorily restoring the staining susceptibility of osmium material by means of sulphurous acid (obtained by adding hydrochloric acid to bisulphite of sodium, 2 to 4 drops of the acid added to 10 c.c. of a 2 per cent. solution of the salt).

FOL (*Lehrb.*, p. 174) recommends a weak aqueous solution of ferricyanide of potassium.

Lee finds that sulphate of iron solution used in Benda's hæmatoxylin stain has a marked bleaching effect, and so also, though in a less degree, the iron alum of Heidenhain's process.

ALTMANN (*Die Elementarorganismen*, pp. 33 and 35) puts sections overnight into gold chloride of 2 per cent., and reduces in formic acid in the sun, and removes the gold by iodised alcohol.

But perhaps the best plan is the chlorine method of MAYER, or his magnesium peroxide, for both of which see "Bleaching."

The same stains recommended for objects fixed by the vapours will be found useful here. For sections, of course, in both cases safranin and other anilin stains may be employed with advantage, as may hæmatoxylin.

In general, osmic acid, especially when used in the form of vapour, fixes protoplasm very faithfully, nuclei badly. It is pre-eminently a fixative of the *hyaloplasm* or enchylema of cells. The *penetrating power* of the solution is *very low*, so that if any but very small pieces of tissue be taken the outer layers become over-fixed before the reagent has penetrated to the deeper layers. Over-fixed cells have a certain homogeneous, glassy, or colloid look, and are unfit for study, and attention should be confined to cells four or five layers deeper down, which will generally be found to present the required intensity of fixation. In these the fixation is admirable, with no shrinkage and next to no swelling of anything. See also § 31.

41. The Osmium Tetroxide Reaction. MANN believed that during the osmic reaction on fatty substances the OsO_4 was reduced to osmium tetra-hydroxide $Os(OH)_4$. Other observers have assumed the reaction to be the reduction of the OsO_4 to some lower oxide. The matter has recently been reviewed by Professor J. R. PARTINGTON and Mr. D. B. HUNTINGFORD who, find that the reduced substance is a hydrated form of OsO_2. possibly OsO_2, $5H_2O$, or OsO_2, $6H_2O$. In all probability,

Professor Partington informs us, the amount of water is not definite.

Estimation of Osmium Tetroxide. Tschugaeff (*C. R. Acad. Sci.*, 167, 1918) discovered a test for concentration of osmic acid, which has been discussed and usefully amplified by Richard Palmer (*J.R.M.S.*, 1930). A solution of OsO_4, heated with thiourea in excess, with a few drops of HCl diluted to $\frac{1}{5}$ in. strength, gives a clear red colour, varying in depth with the concentration of osmic acid used.

42. Osmic Mixtures. Nicolas (*Intern. Monatsschr.*, 1891, p. 3) adds $\frac{1}{2}$ per cent. of osmic acid to nitric acid of 3 per cent. Lee has employed a similar mixture and not had good results, though he found the mixture kept perfectly.

Busch (*Neurol. Centralb.*, xvii, 1898, No. 10, p. 476 ; *Zeit. wiss. Mik.*, xv, p. 373) finds that the penetration of osmic acid is enhanced by combining it with iodate of sodium, which by hindering its too rapid decomposition in the tissues ensures a more energetic action in the deeper layers. He adds 3 per cent. of sodium iodate to a 1 per cent. solution of osmic acid.

Unna (*Monatsschr. prakt. Derm.*, xxvi, 1898, p. 602) adds 1 per cent. of alum to a 1 per cent. solution. For some mixtures of Kolossow, see *5th ed.*, or *Zeit. wiss. Mikr.*, v, 1888, p. 51, and ix, 1892, p. 39.

43. Chromic Acid. Chromic anhydride, CrO_3, is found in commerce in the form of red crystals that dissolve readily in water, forming chromic acid, H_2CrO_4. These crystals are very deliquescent, and it is therefore well to keep the acid in stock in the shape of a 1 per cent. solution. Care must be taken not to allow the crystals to be contaminated by organic matter, in the presence of which the anhydride is readily reduced into sesquioxide.

Chromic acid is generally employed in aqueous solution. Some observers (Klein ; Urban Pritchard ; Perényi) have recommended alcoholic solutions ; but this is evidently irrational. For in the presence of alcohol chromic acid has a great tendency to *become reduced* to chromous oxide or sesquioxide, neither of which appears to have any fixing power.

The most useful strengths in which it is employed in aqueous solution are from 0·1 to 1·0 per cent. for a period of immersion of a few hours (structure of cells and ova). For nerve tissues weaker solutions are taken, $\frac{1}{50}$ to $\frac{1}{8}$ per cent. for a few hours. Stronger solutions, such as 5 per cent., should only be allowed to act for a few seconds.

Washing out. The general practice is to wash out very thoroughly with water (by preference running water, for many hours) before bringing into alcohol or any staining liquid. For if the objects are put direct into alcohol it is found that after a short time a fine precipitate is thrown down on the surface of the preparations, thus forming an obstacle to the further penetration of the alcohol. Previous washing by water does not prevent the

formation of this precipitate, and changing the alcohol does not prevent it from forming again and again. It has, however, been found by HANS VIRCHOW (*Arch. mik. Anat.*, xxiv, 1885, p. 117) that it may be entirely prevented by simply keeping the preparations *in the dark.* The alcohol becomes yellow as usual (and should be changed as often as this takes place), but no precipitate is formed. If this precaution be taken, previous washing with water may be omitted, or at all events greatly abridged.

MAYER (*Grundzüge*, 1st ed., p. 28) proceeds as follows :—The fixed material is merely rinsed in water and brought direct into 70 per cent. alcohol. It is washed therein, preferably in the dark, until after several changes the alcohol remains colourless. It is then either passed through higher alcohols and imbedded in paraffin, the chromous oxide (or whatever chrome compound it may be that is present in the tissues) being removed from *the sections* after these are made ; or this necessary removal is performed at once. If this be preferred, the material is brought into sulphuric acid diluted with twenty volumes of water, or into nitric acid diluted with ten volumes of water. After at most a few hours therein, it will have become of a light greyish green, and on removal of the acid may be readily stained. If it be preferred to treat *the sections*, it is sufficient to put them into the usual hydrochloric acid alcohol (4 to 6 drops of HCl to 100 c.c. of 70 per cent. alcohol), in which after a short time they become almost white, and will stain excellently with any of the usual stains. See also EDINGER (*Zeit. wiss. Mik.*, i, 1884, p. 126 ; nitric acid 1 : 20 for five minutes). UNNA (*Arch. mik. Anat.*, xxx, 1887, p. 47) holds that the chrome is present in the tissues in the form of chromium chromate, and removes it by treatment with peroxide of hydrogen. OVERTON (*Zeit. wiss. Mik.*, vii, 1890, p. 9) employs a weak solution of sulphurous acid, which converts it into a sulphate. See also the directions for bleaching osmic acid preparations.

Tissues that have been fixed in chromic acid may be stained in aqueous solutions, as water does not have an injurious effect on them.

The best stain for chromic material that has not been treated by Mayer's special process, or by a similar one, is hæmatoxylin, or, for sections, the basic tar colours.

Chromic acid is not a very penetrating reagent, and for this reason, as well as for others, is now seldom used *pure* for *fixing.*

For prolonged hardening it is generally employed in strengths of $\frac{1}{5}$ to $\frac{1}{2}$ per cent., the immersion lasting a few days or a few weeks, according to the size and nature of the object. Mucous membrane, for instance, will harden satisfactorily in a few days ; brain will require some six weeks.

Large quantities of the solution must be taken (at least 200 grm. for a piece of tissue of 1 cm. cube—Ranvier).

In order to obtain the best results you should not employ portions of tissue of more than an inch cube. For a human spinal cord you should take 2 litres of solution, and change it for

fresh after a few days. Six weeks or two months are necessary to complete the hardening.

Lee thought it was frequently useful to *add a little glycerin*; there is then less brittleness.

The solution should be taken weak at first, and the strength increased after a time. The objects should be removed from the solution as soon as they have acquired the desired consistency, as if left too long they will become brittle. They may be preserved till wanted in alcohol (95 per cent.). It is well to wash them out in water for twenty-four or forty-eight hours before putting them into the alcohol. After a time they generally become green in the alcohol. They may be *bleached* if desired.

Chromic acid is a most powerful and rapid hardening agent. (By it you may obtain in a few days a degree of hardening that you would hardly obtain in as many weeks with bichromate for instance.) It has the defect of a great tendency to cause *brittleness*.

44. Chromo-acetic Acid (Flemming, *Zellsbz., Kern. u. Zellth.*, p. 382).

Chromic acid . .	0·2 to 0·25 per cent. in water with,
Acetic acid . .	0·1 per cent.

Flemming found this the best reagent for the study of the *achromatic* elements of karyokinesis. You can stain with hæmatoxylin, or the basic anilin dyes.

The following has been recommended for Annelids by Ehlers :—To 100 c.c. of chromic acid of 0·5 to 1 per cent. add from 1 to 5 drops of glacial acetic acid. The acetic acid is said to be sufficient to counteract any shrinkage due to the chromic acid. Fix overnight, wash out several hours in water.

Similar to this is the "chromo-acetic acid, No. 1," of Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443), viz. 1 part 50 per cent. acetic acid and 20 parts 1 per cent. chromic acid, which is found very useful for fixing marine animals.

45. Chromo-nitric Acid (Perényi's formula, *Zool. Anzeig.*, v, 1882, p. 459).

4 parts 10 per cent. nitric acid.
3 parts alcohol.
3 parts 0·5 per cent. chromic acid.

These are mixed, and after a short time give a fine violet-coloured solution.

The objects are immersed for four to five hours, and then passed through 70 per cent. alcohol (twenty-four hours) strong alcohol (some days), absolute alcohol (four to five days). They are then fit for cutting. The advantage of the process is, amongst others, that segmenting eggs and nuclei are perfectly fixed, the ova do not become porous, and cut like cartilage.

Chromo-nitric acid is not only an embryological reagent, and a very important one, but also an admirable one for general

work. Lee found it altogether excellent for preserving marine organisms, especially large forms. Strong alcohol need only be used if the objects are destined to be sectioned.

Another advantage is that the fixing solution may be combined with a stain. (In this case the albuminous envelopes of the ova must be carefully removed, otherwise the stain will not penetrate.)

Some stains, such as fuchsin or anilin red, may be dissolved directly in the fixing solution. Others, such as eosin, purpurin, anilin violet, must first be "dissolved in three parts of alcohol, and then shaken into the liquid."

Picro-carmine and borax-carmine may be added to the liquid, but they give rise to a precipitate, which must be removed by filtration before using.

Another formula given by Perényi (*Zool. Anzeig.*, 274, 1888, p. 139, and 276, p. 196) is as follows :

3 parts 20 per cent. nitric acid.
3 parts 1 per cent. chromic acid.
4 parts absolute alcohol.

For embryos of Lacerta. Fix for twenty minutes. Wash out for an hour with 70 per cent. alcohol, and then with strong alcohol. Stain with Delafield's hæmatoxylin and treat the stained material for three to five minutes in 1 per cent. chromic acid.

46. **Chromo-formic Acid** (Rabl, *Morph. Jahrb.*, x, 1884, pp. 215, 216). Four or 5 drops of concentrated formic acid are added to 200 c.c. of 0·33 per cent. chromic acid solution. The mixture must be freshly prepared at the instant of using. Fix for twelve to twenty-four hours, wash out with water. Used by Rabl for the study of karyokinesis. (See also under Formic Acid, § 123.)

47. **Chromo-aceto-osmic Acid** (Flemming, *Zellsubstanz, Kern und Zelltheilung*, 1882, p. 381). First or Weak formula :

Chromic acid . . .	0·25 per cent.	in water.
Osmic acid . . .	0·1 ,,	
Glacial acetic acid . .	0·1 ,,	

Meves (*Encycl. mikr. Techn.*, 1, p. 475) sometimes added 1 per cent. of sodium chloride. See also § 31.

Fol (*Lehrb. d. vergl. mik. Anat.*, 1884, p. 100) recommends the following variant :

1 per cent. chromic acid	25 vols.
1 per cent. osmic acid	2 ,,
2 per cent. acetic acid	5 ,,
Water	68 ,,

—that is to say, a mixture weaker in osmium than Flemming's.

A mixture still weaker than this in osmium, viz. with 1 vol. osmic acid solution, instead of 2, has been recommended by Cori (*Zeit. wiss. Mik.*, vi, 1890, p. 441).

Second or Strong formula (*Zeit. wiss. Mik.*, 1, 1884, p. 349) :

1 per cent. chromic acid	15 parts.
2 per cent. osmic acid	4 ,,
Glacial acetic acid	1 part.

If this mixture be kept in stock in large quantities, it may go bad, on account of the large proportion of organic acid contained in it. We therefore recommend that the osmic and chromic acid be kept ready mixed in the proportions given, and 5 per cent. of acetic acid added at the moment of using.

It has been pointed out by J. Z. Young (*Nature*, May 18th, 1935) that the addition of 0·75 to 0·95 per cent. NaCl, according as to whether cold-blooded or warm-blooded animal tissues are being fixed, appears to increase the area of cells fixed. In experiments we have made Young's claim appears to be substantiated, and we strongly recommend a trial of this modification. It should be mentioned that Meves sometimes added 1 per cent. NaCl to the weak formula. In our experience the addition of NaCl causes disintegration of the solution in only a few weeks, and the fluid if to be stored should be made up as follows: solution A, 15 parts of 1 per cent. chromic acid in aq. dest.; solution B, 4 parts of 2 per cent. OsO_4 in 4 per cent. (actually 4·275 per cent.) NaCl. Dissolve the osmic first and then add the salt. Before fixing material add 15 parts of A to 4 parts of B. This gives a Flemming without acetic acid with 0·9 NaCl.

Weaker Formula. Later, Flemming has been making up the mixture with only 2 parts of the osmic acid instead of 4, and has spoken of this modification as "weaker osmium mixture" (Meves, in *Encycl. mikr. Techn.*, p. 476).

Meves (*loc. cit.*) takes for *delicate objects* 15 parts of chromic acid of only 0·5 per cent., 2 or 4 of osmic acid of 2 per cent., and 1 of acetic acid, and thus gets less shrinkage.

Under "Cytology" Sections, see Benda, Gatenby, and Guthrie modifications (§ 679).

Podwyssozki recommends (for glands especially) the following modification:

1 per cent. CrO_3 dissolved in 0·5 per cent. solution of corrosive sublimate	15 c.c.
2 per cent. osmic acid solution	4 c.c.
Glacial acetic acid	6 to 8 drops.

The sublimate is said to augment the penetration of the osmium, but is unfavourable to staining (Ziegler's *Beiträge z. path. Anat.*, i, 1886; *Zeit. wiss. Mik.*, iii, 1886, p. 405).

The first or weak liquid is the better for very small objects, the second or strong one for larger ones, as it has *better penetration.* These liquids may be allowed to act for many hours or days or according to some workers even weeks or months; but this exaggerated fixation is clearly only justifiable in very special cases, if at all. For chromosome studies some workers fix for only one hour. Others recommend cooling the Flemming on ice before using. Wash out very thoroughly in water (running, twenty-four hours), or treat as directed for chromic acid, § 43.

Stain with alum hæmatoxylin if you wish to stain *in toto* (staining in this way with other reagents is possible, but difficult). Stain sections with safranin or other basic coal-tar colour, or with iron hæmatoxylin.

For fixing with the *strong* mixture you need only take a bulk of liquid of some four times the volume of the objects (but with the weak mixture the proportion should be increased). Both of them are first-rate fixatives of cellular structures, both as regards their *preservation* and as regards their *optical differentiation*. But they must be properly used, and not applied to objects for which they are not fitted. For instance, their *power of penetration* is *extremely bad*; they will not fix properly, even in a loose-celled tissue, through more than a layer of about five cells thick. They are therefore suitable only for *very small* objects or for *very small* pieces of tissue, such as suffice for cytological or histological work. As mentioned in § **31** and above, the addition of NaCl to the Flemming does appear to increase the penetration of both this fluid and Champy's mixture. The strong liquid especially has not the character of a *general* reagent. As a matter of fact it was recommended by Flemming in the first instance merely for a very special purpose, the hunting for karyokinetic figures, and not for general purposes. It is still very much used, but in most cases, Bouin's picroformol will do all that it is intended to do, without its disadvantages.

It may be used for prolonged hardening, *e.g.* of small pieces of nervous tissue, and is very good for that purpose.

Fat is blackened (or browned) by it. Chromatin is mordanted by it for the basic anilin dyes, enabling them to give peculiarly sharp and powerful stains.

48. Osmic Acid and Bichromate. Altmann (*Die Elementarorganismen*, Leipzig, 1890) takes for his "bioblasts" a mixture of equal parts of 5 per cent. solution of bichromate of potash and 2 per cent. solution of osmic acid. The bichromate ought not to contain any free chromic acid. Refer to § 694.

Lo Bianco (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) employs for marine animals a mixture of 100 c.c. of 5 per cent. solution of bichromate and 2 c.c. of 1 per cent. osmic acid.

Hoehl (*Arch. Anat. Phys., Anat. Abth.*, 1896, p. 31) recommends a mixture of 80 c.c. of 3 per cent. bichromate, 20 c.c. of 1 per cent. osmic acid, and 2 c.c. of glacial acetic acid.

Bensley (see Cowdry, "Mitochondrial Constituents," *Contrib. Embryol., Carneg. Inst.*, Washington) uses 2·5 per cent. bichromate.

Baker and Thomas take equal parts of 3 per cent. bichromate and 2 per cent. osmium. See "Cytological Technique" (Baker).

49. Bichromate-chromic-osmic Acid. Champy (*Arch. de Zool. Expér.*, 1913). Mixture of 7 parts of 3 per cent. bichromate of

potash, 7 parts of 1 per cent. chromic acid, 4 parts of 2 per cent. osmium tetroxide.

This mixture keeps well. Fix for from six to twenty-four hours. Wash out in running water about the same time.

You can stain in iron hæmatoxylin, or less well in ALTMANN or BENDA. See § 706 for a description of mordanting after Champy's fluid. This fluid is extremely useful, and we nearly always use it in addition to FLEMMING.

Since the addition of 0·75 per cent. to 0·9 per cent. NaCl causes disintegration of this solution in about a week, the salt Champy may be made up as follows. Seven parts of 3 per cent. bichromate, 7 parts of 1 per cent. chromic; then add before use 4 parts of 2 per cent. osmium in 4 per cent. NaCl (actually 4·275 per cent.) to make a Champy with 0·9 per cent. NaCl. Dissolve the osmic acid first, and then the NaCl.

50. Osmic, Bichromate, and Platinic Mixture (LINDSAY JOHNSON'S Mixture).

Bichromate of potash (2·5 per cent.) .	70 parts.
Osmic acid (2 per cent.) . . .	10 „
Platinum chloride (1 per cent.) . .	15 „
Acetic or formic acid	5 „

HENNEGUY, who has worked a great deal with this reagent, and recommended it highly, says (*Leçons sur la Cellule*, p. 61) that it is well only to add the acetic or formic acid just before using, as it frequently reduces the osmium and platinum very rapidly and energetically. He finds that it contracts the more spongy sorts of protoplasm less than mixture of FLEMMING. Lee thought highly of it—for certain objects. Twelve hours is probably the optimum time for fixation. Wash out in water.

51. Platino-aceto-osmic Acid (HERMANN'S) **Solution** (*Arch. Mik. Anat.*, xxxiv, 1889, p. 58). One per cent. platinum chloride 15 parts glacial acetic acid 1 part, and 2 per cent. osmic acid either 4 parts or only 2 parts. Hermann found that protoplasm structures are thus better preserved than with the chromic mixture. As with Flemming, the optimum time is from twelve to sixteen hours. Wash out at least three hours in running water.

The after-treatment and staining should be the same as for objects treated with Flemming's solution. RENGEL (*Zeit. wiss. Zool.*, lxiii, 1898, p. 454) washes out for half an hour to an hour with saturated aqueous sol. of picric acid, which he thinks facilitates the staining especially of nuclei.

The action of this fixative is, roughly, similar to that of Flemming's. Like Flemming's, it mordants chromatin for staining with "basic" colours, with which it affords equally fine nuclear stains. But, owing to the platinum in it, it diminishes more than Flemming's the colorability of tissues with "acid" colours, so that it is *extremely difficult* to obtain *good plasma stains* after its action. It causes a notable shrinkage in chromatin. It gives a *full* fixation of cytoplasm, to which it gives a much more fine-grained aspect than liquid of Flemming does.

Leaving out the acetic acid, the solution may be used for mitochondria as in § 693.

52. Rawitz (*Zeit. wiss. Mikr.*, xxv, 1909, p. 386) takes 4 parts of Kahlbaum's **Phospho-Tungstic** acid, 5 of alcohol, and 1 of acetic acid,

added just before use, fixes for twenty-four hours, and washes out the sections before staining with water containing a little calcium acetate.

53. Nitric Acid (ALTMANN, *Arch. Anat. Phys.*, 1881, p. 219). Altmann employs for fixing embryos dilute nitric acid, containing from 3 to 3½ per cent. pure acid. Such a solution has a sp. gr. of about 1·02. Stronger solutions have been used, but do not give such good final results. After extensive trial Lee found ALTMANN's solution to be a second-rate reagent, giving a weak and thin fixation.

HIS (*ibid.*, 1877, p. 115) recommended a 10 per cent. solution. Flemming at one time employed solutions of 40 to 50 per cent. for the ova of Invertebrates.

TELLYESNICZKY (*Arch. mik. Anat.*, lii, 2, 1898, p. 222) thinks that "for general cell-fixing" the proper strength is 2 to 2½ per cent., as stronger grades act too energetically on the superficial layers.

MAYER has had good results with 5 per cent. solution.

Nitric acid has the valuable property of *hardening yolk without making it brittle.*

Pure water should in no case be used for washing out; the preparations should be brought direct into alcohol. Some persons take absolute, but 70 per cent. is more generally indicated. Rabl has employed a 1 or 2 per cent. solution of alum.

For *prolonged hardening*, strengths of from 3 to 10 per cent. are sometimes employed. A strength of 12 per cent., allowed to act for two or three weeks, is said to afford very tough preparations of the encephalon.

BENDA (*Verh. Anat. Ges.*, 1888; *Ergeb. d. Anat.*, i, 1891, p. 7) fixes for twenty-four to forty-eight hours in 10 per cent. nitric acid, and then hardens in bichromate of potash.

Fol's Mixture (verbally communicated to Lee). Three vols. of nitric acid, with 97 vols. of 70 per cent. alcohol.

54. Chromic Acid and Platinum Chloride (MERKEL's *Macula lutea des Menschen*, Leipzig, 1870, p. 19). Equal volumes of 1·400 solution of chromic acid and 1·400 solution of platinum chloride. Objects should remain in it for several hours or even days. After washing out with alcohol of 50 to 70 per cent., objects stain excellently. If objects that have been fixed by osmic acid be put into it for some hours, blackening is said to be effectually prevented.

This is an excellent hardening medium for delicate objects. Merkel allowed from three to four days for the action of the fluid for the retina; for Annelids Eisig employs an immersion of three to five hours, and transfers to 70 per cent. alcohol; for small leeches Whitman finds one hour sufficient, and transfers to 50 per cent. alcohol.

A similar mixture, with the addition of 0·25 to 0·1 per cent. of acetic acid, is recommended by BRASS for Protozoa; and LAVDOWSKY has used for nuclei a mixture of 10 parts of 1 per cent. chromic acid, 5 of 1 per cent. platinum chloride, and 100 of 5 cent. acetic acid.

Whitman recommends for the hardening of pelagic fish ova a stronger mixture (due, we believe, to Eisig), viz.—

0·25 per cent. solution of platinum chloride .	1 vol.
1 per cent. solution of chromic acid . .	1 ,,

The ova to remain in it one or two days (WHITMAN, *Methods in Micro. Anat.*, p. 153).

SALTS

55. Chromates. The chromates are amongst the oldest and best tried of hardening agents. The bichromate of potash

especially was at one time universally employed for hardening all sorts of tissues.

Flemming (*Arch. mik. Anat.*, xviii, 1880, p. 352) pointed out that though it preserves cytoplasm well it causes chromatin to swell, and therefore should not be employed *for the study of nuclei.* But, *duly corrected with acetic acid,* it affords a correct and fine fixation of nuclei; whilst preserving secretions, etc., much better than chromic acid.

For general work Kultschitzky and Zenker or Zenker Formalin are the only bichromate formulæ which are recommendable, and probably only for vertebrate material. The chromates are of great importance in neurological work, and as fixing fluids before staining bacteria in tissues.

None of these solutions approaches Bouin's fluid in general applicability, Tellyesniczky and Müller usually giving atrocious results.

For an elaborate study of the action of chrome salts on nucleus and cytoplasm, see Burckhardt, *La Cellule*, xii, 1897, p. 335.

For the demonstration of the achromatic figure of cell division he recommends—

Chromic acid, 1 per cent. solution . . .	60 vols.
Bichromate of potassium, 5 per cent. solution .	30 ,,
Glacial acetic acid	5 ,,

56. Bichromate of Potash.* Perhaps the most important of all known *hardening* agents, *senu stricto.* It hardens slowly, much more so than chromic acid, but it gives an incomparably better consistency to the tissues. They may remain almost indefinitely exposed to its action without much hurt.

The strength of the solutions employed is from 2 to 5 per cent. As with chromic acid, it is extremely important to begin with weak solutions and proceed gradually to stronger ones. About three weeks will be necessary for hardening a sheep's eye in solutions gradually raised from 2 to 4 per cent. Spinal cord requires from three to six weeks, a brain at least as many months.

After hardening, the objects should be well soaked out in water before being put into alcohol, or be treated as directed for chromic acid, § 43. They had better be kept in the dark when in alcohol. See § 43. (Böhm and Oppel [*Taschenbuch*, 3 *Auf.*, 1896, p. 22] fix in the dark.) *If you wish to have a good stain with carmine you should not put the objects into alcohol at all, even for a second, until they have been stained.*

You may stain either with carmine or hæmatoxylin, as well as with tar colours.

Bichromate objects have an ugly yellow colour which cannot be removed by mere soaking in water. It is said that it can be removed by washing for a few minutes in a 1 per cent. solution of chloral hydrate.

Prof. Gilson wrote that alcoholic solution of sulphurous anhydride (SO_2) is very convenient for the rapid decoloration of bichromate objects. A few drops suffice. See also § 43, and "Bleaching."

To facilitate staining with hæmatoxylin, Wolff (*Zeit. wiss. Mik.*, xv, 3, 1899, p. 311) first stains in Boehmer's hæmatoxylin for twenty-four hours, and then for a few minutes in the same hæmatoxylin to which

* Regaud's fluid, § 699.

has been added 1 drop per watch-glassful of 5 per cent. solution of oxalic acid.

The simple aqueous solution of bichromate is hardly to be recommended as a *fixing* agent, because not only does it not preserve nuclei properly, but also because it penetrates very slowly. The first of these defects may be overcome entirely, the second to some extent by addition of acetic acid ; whence the liquid of TELLYESNICZKY, next §.

57. Acetic Bichromate (TELLYESNICZKY, *Arch. mik. Anat.*, lii, 1889, p. 242) :

Bichromate	3 grm.
Glacial acetic acid	5 c.c.
Water	100 ,,

Smaller objects to remain in the fluid for one or two days, larger ones longer. Wash well in plenty of water, and pass through alcohols of increasing strengths, beginning with 15 per cent.

Mixtures of bichromate with osmic acid have been given above, §§ 48 *et seq.*

58. MÜLLER'S Solution.

Bichromate of potash	2—2½ parts.
Sulphate of soda	1 part.
Water	100 parts.

The duration of the reaction is about the same as with the simple solution of chromic salt.

Recent authors find the action of this liquid to be identical with that of plain bichromate, and doubt whether the sulphate in it has any effect whatever as regards its hardening properties. Fol says that for mammalian embryos, for which it has been recommended, it is worthless.

59. ERLICKI'S Solution (*Warschauer med. Zeit.*, xxii, Nos. 15 and 18 ; *Progrès Médical*, 1897, No. 31) :

Bichromate of potash	2·5 parts.
Sulphate of copper	1·0 part.
Water	100·0 parts.

Here the addition of the cupric sulphate is intelligible, for this salt is itself a hardening agent of some energy. As a matter of fact, " Erlicki " hardens very much more rapidly than either simple bichromate or Müller's solution. A spinal cord may be hardened in it in four days at the temperature of an incubator (30°—40° C.), and in ten days at the normal temperature (FOL, *Lehrb. d. vergl. mik. Anat.*, p. 106). Human embryos of several months may be conveniently hardened in it.

Nerve-centres that have been hardened in Erlicki's fluid frequently contain dark spots with irregular prolongations, simulating ganglion-cells. These are now known to consist of precipitates formed by the fluid. They may be removed by washing with hot water, or with water slightly acidified with hydrochloric acid, or by treating the specimens with 0·5 per cent. chromic acid before putting them into alcohol (TSCHISCH, *Virchow's Arch.*, Bd. xcvii, p. 173 ; EDINGER, *Zeit. wiss. Mik.*, ii, p. 245 ; LOEWENTHAL, *Rev. méd. de la Suisse romande*, 6me année, i, p. 20).

60. KULTSCHITZKY'S Solution (*Zeit. wiss. Mik.*, iv, 1887, p. 348). A saturated solution of bichromate of potash and sulphate of copper in

50 per cent. alcohol, to which is added at the instant of using a little acetic acid, 5 or 6 drops per 100 c.c.

To make the solution, add the finely powdered salts to the alcohol in excess, and leave them together *in total darkness*, for twenty-four hours.

Fix for twelve to twenty-four hours *in the dark*. Then treat with strong alcohol for twelve to twenty-four hours.

61. Dekhuyzen's **Liquids** (*C. R. Acad. Sci.*, cxxxvii, 1903, pp. 415 and 445). (A) 250 c.c. of 2·5 per cent. sol. of bichromate in sea-water, 25 c.c. of 6·3 per cent. nitric acid, and 54 c.c. of 2 per cent. osmic acid. For general use with marine animals.

(B) 173·1 c.c. of the bichromate sol. and 26·9 of 2 per cent. sol. of osmic acid.

For objects containing calcareous elements that it is desired to preserve.

These liquids are stated to be isotonic with sea-water.

62. **Bichromate and Sublimate** (Kultschitzky, *Arch. f. mik. Anat.*, xlix, 1897, p. 8). Two grm. bichromate, ¼ grm. corrosive sublimate, 50 c.c. 2 per cent. acetic acid, and 50 c.c. 96 per cent. alcohol. The mixture should be filtered after twenty-four hours. Tissues of vertebrates may remain in it for four to six days. Lavdowsky (*Zeit. wiss. Mik.*, xvii, 1900, p. 301) takes 500 c.c. of 1 per cent. acetic acid, 20 to 25 grm. bichromate, and 5 to 10 c.c. saturated solution of sublimate in water. See Zenker, § 78.

63. **Bichromate of Ammonia.** This salt is in considerable favour for hardening. Its action is very similar to that of the potassium salt. Fol says that it penetrates somewhat more rapidly, and hardens somewhat more slowly. It should be employed in somewhat stronger solutions, up to 5 per cent.

64. **Neutral Chromate of Ammonia** is preferred by some. It is used in the same strength as the bichromate. Klein has recommended it for intestine, which it hardens, in 5 per cent. solution, in twenty-four hours.

65. **Bichromate of Calcium.** Sonnenbrodt (*Arch. mikr. Anat.*, lxxii, 1908, p. 416) fixes ovaries of *Gallus* in 20 parts of 2 per cent. sol. of calcium bichromate with 10 of 2 per cent. sol. of sublimate and 1 of acetic acid.

66. **Bichromates and Alcohol.** Mixtures of bichromate of potash or ammonia with alcohol may be employed, and have a more rapid action than the aqueous solution. Thus Hamilton takes for hardening brain a mixture of 1 part methylated spirits with 3 parts of solution of Müller; see also Kultschitzky's Mixture, *ante*, § 60). Preparations should be kept in the dark during the process of hardening in these mixtures.

67. **Sulphurous Acid.** Waddington (*Journ. Roy. Mic. Soc.*, 1883, p. 185) uses a saturated solution of sulphurous acid in alcohol for fixing infusoria. Overton (*Zeit. wiss. Mik.*, vii, 1890, p. 9) uses the vapours of an aqueous solution for fixing algæ.

CHAPTER V

FIXING AND HARDENING AGENTS—CHLORIDES, ORGANIC ACIDS, AND OTHERS

CHLORIDES

68. Bichloride of Mercury (Corrosive Sublimate). Corrosive sublimate 1 part is soluble in about 16 parts of cold and 3 of boiling distilled water. It is more soluble in alcohol (1 : 3) or in ether (1 : 4) than in water. Its solubility in all these menstrua is augmented by the addition of hydrochloric acid, ammonium chloride, or camphor. With sodium chloride it forms a more easily soluble double salt; hence sea-water may dissolve over 15 per cent.

The simple aqueous solutions should always be made with *distilled*—not spring—water. The $HgCl_2$ in them has been assumed partly to split up by hydrolysis into Cl, H, and $(HgCl)_2$, or HgClOH (see *Chem. Centralb.*, 1904, i, p. 571). But J. Baker (*Cytological Technique*, 1933) points out that mercuric chloride partly hydrolyses into hydrogen and chlorine ions and $(HgCl)_2O$ or HgClOH, according to Luther (*Chem. Centralbl.*, 8, i, 1904). These solutions should give an acid reaction with litmus paper, whilst those made with strong sodium chloride solution are neutral.

Carleton (*Q. J. M. S.*, vol. lxvi, 1922) has investigated the comparative effects of isotonic saline and distilled water when used as solvents for mercuric chloride and formol in histological fixation. From this work it appears to be of no histological importance whether saturated (6 per cent.) solutions of mercuric chloride be dissolved in normal saline or in distilled water. No differences could be detected in specimens of liver, small intestine, and kidney fixed in either way, nor would there be any reason to expect such differences on *a priori* grounds. For the relatively high molecular concentration of the $HgCl_2$ is only very slightly altered by dissolving it in either isotonic saline or in hypertonic saline of double the normal concentration. In fact, the only effect of making up a concentrated solution of mercuric chloride in normal saline is slightly to increase the tonicity of the mixture. There is still (1937) a question of the exact molecular condition of fixing fluids with NaCl which has been raised again by J. Z. Young (§ 31), and the question will have to be reviewed in the future.

In the case of a 5 per cent. solution of formol the evidence is that this reagent fixes tissues more faithfully when made up in normal saline than in distilled water. When dissolved in the

latter the ground cytoplasm is often vacuolated, and sometimes partly destroyed.

For fixing, corrosive sublimate may be used pure; but in most cases a finer fixation will be obtained if it be *acidified with acetic acid*, say about 1 per cent. of the glacial acid. We find that a saturated solution in 5 per cent. glacial acetic acid is a very good formula for *marine* animals; for others take the acid weaker. KAISER'S solution consists of 10 grm. sublimate, 3 c.c. glacial acetic acid, and 300 c.c. distilled water (from *Zeit. wiss. Mik.*, xi, p. 378). VAN BENEDEN has used a saturated solution in 25 per cent. acetic acid, and LO BIANCO (*Mitth. Zool. Stat. Neapel*, ix, 1890, p. 443) a mixture of 2 parts saturated solution with 1 part of 49 per cent. acetic acid.

It is sometimes advisable to take the most concentrated solution obtainable. For some very contractile forms (coral polypes, Planaria), a concentrated solution in *warm* or even boiling water should be employed. For Arthropoda *alcoholic* solutions are indicated. Delicate objects, however, may require treatment with weak solutions.

Objects should in all cases be removed from the fixing bath *as soon as fixed*, that is, as soon as they are seen to have become opaque throughout, which may be in a few minutes or even seconds.

Wash out with water or alcohol. *Alcohol is almost always preferable.* Alcohol of about 70 per cent. may be taken, and (MAYER, *Intern. Monatsschr. Anat. Phys.*, iv, 1887, p. 43) a little tincture of iodine * may be added to the liquid, either alcohol or water, used for washing, enough to make it of a good port-wine colour, and the mixture be changed until it no longer becomes discoloured by the objects. APÁTHY (*Mikrotechnik*, p. 148) takes a 0·5 per cent. solution of iodine in strong alcohol, leaves the objects in it (suspended) until they have become of about the same colour as the solution, and then washes for twenty-four hours in pure alcohol.

J. BAKER (*op. cit.*) states that the precipitate is probably either mercurous chloride, or a phosphate formed by reaction with the phosphates found in cells.

In *obstinate cases* solution of iodine in iodide of potassium (*e.g.* LUGOL'S) may be taken. MAYER (*Zeit. wiss. Mik.*, xiv, 1897, p. 28) makes it by dissolving 5 grm. of iodide of potassium in 5 c.c. of distilled water and mixing this with a solution of 0·5 grm. of iodine in 45 c.c. of 90 per cent. alcohol, but seldom uses the mixture concentrated, merely adding as much of it as is required to the alcohol or water containing the objects. The important point is, that the iodine and iodide be employed together. The iodine may be washed out in obstinate cases with magnesia water. Similarly APÁTHY (*Mitth. Zool. Stat. Neapel*, xii, 1897, pp. 729, 730).

It has been objected to this process that iodine in potassium iodide precipitates corrosive sublimate instead of dissolving it. That is true, but the precipitate is soluble in excess of the precipitant.

* Refer to, and contrast, Susa, § 93.

The iodide of potassium process should be employed with care, for the iodide may partly redissolve the precipitated compounds formed by the sublimate with the albumens, etc., of the tissues, and it may be well not to begin adding the iodine till the objects have been brought into fairly strong alcohol, 70 or 80 per cent.

It is important that the sublimate be *thoroughly* removed from the tissues, otherwise they become *brittle*, and will not stain so well. They may also become brittle if they are kept long in alcohol.

It may happen that if the extraction of the excess of sublimate from the tissues in bulk has been insufficient, crystals may form in the sections after they have been mounted in balsam. This may easily be prevented by treating the sections themselves with tincture of iodine for a quarter of an hour before mounting. Some workers hold that this does away with the necessity of treating the tissues in bulk with iodine, which is frequently a very long process. Thus, MANN (*Zeit. wiss. Mik.*, xi, 1894, p. 479) prefers treating the sections rather than the tissues in bulk, on the ground that the iodine makes them soft, so that they shrink on coming into paraffin. SCHAPER (*Anat. Anz.*, xiii, 1897, p. 463), *however, has shown that neglect to extract the sublimate from the tissues in bulk may give birth to serious artifacts, which appear to arise during the imbedding process.* So also LOYEZ (*Arch. Anat. Micr.*, viii, 1905, p. 71). HEIDENHAIN (*Zeit. wiss. Mik.*, xxv, 1909, p. 398) removes the iodine from sections by means of sodium thiosulphate.

You may stain in any way you like. Carmine stains are peculiarly brilliant after sublimate.

The solutions must not be touched with iron or steel, as these produce precipitates that may hurt the preparations. To manipulate the objects, wood, glass or platinum may be used; for dissecting them, hedgehog spines, or quill pens, or cactus spines.

When properly employed, sublimate is *for general work* undoubtedly a most useful fixing agent. It is applicable to most classes of objects. It is perhaps less applicable, in the pure form, to Arthropods, as it possesses no great power of penetrating chitin. For *cytological work* it is, according to our experience, not to be trusted unless with osmic acid, and only to be recommended where more precise fixing agents are contra-indicated by reason of their lack of penetration, or the like. Amongst other defects it has that of frequently causing *very serious shrinkage* of cells.

69. Sublimate with Salt. A solution containing 5 grms. sublimate, 0·5 grm. sodium chloride, and 100 c.c. water has been quoted as "solution of GAULE."

A ½ per cent. aqueous solution of sodium chloride saturated whilst hot with sublimate was much recommended by HEIDENHAIN (*Festschrift f. Koelliker*, 1892, p. 109).

The addition of sodium chloride allows a stronger solution to be obtained than can be made with pure water, and also, it is stated, enhances the penetration of the sublimate. But the fixation-precipitates (§ 28) formed by the double salt are (according to SPULER, *Encyl. mik. Technik.*, p. 1274) for the most part soluble in water, thus giving rise to imperfect preservation.

Concentrated (*i.e.* over 20 per cent.) solution in sea-water is recommended for some marine animals.

STOELZNER (*Zeit. wiss. Mikr.*, xxiii, 1906, p. 25) recommends saturated solution of sublimate in sugar solution of 4½ per cent., as isotonic (for warm-blooded animals).

Liquid of Lang (*Zool. Anzeiger*, 1878, i, p. 14). For *Planaria.*

Distilled water	100 parts.
Chloride of sodium	6 to 10 ,,
Acetic acid	6 to 8 ,,
Bichloride of mercury	3 to 12 ,,
(Alum, in some cases	½ part.)

70. Alcoholic Solutions. APÁTHY (*Mikrotechnik*, p. 111) recommends a solution of 3 to 4 grm. of sublimate and 0·5 grm. sodium chloride in 100 c.c. of 50 per cent. alcohol for *general* purposes.

OHLMACHER (*Journ. Exper. Medicine*, ii, 6, 1897, p. 671) takes—

Absolute alcohol	80 parts.
Chloroform	15 ,,
Glacial acetic acid	5 ,,
Sublimate to saturation (about 20 per cent.).	

"Ordinary pieces" of tissue are sufficiently fixed in fifteen to thirty minutes. Entire human cerebral hemispheres, subdivided by Meynert's section, take eighteen to twenty-four hours.

For liquids containing a much higher proportion of acetic acid, see *Acetic Alcohol.*

71. Acetone Solution. HELD (*Arch. Anat. Phys.*, Anat. Abth., 1897, p. 227) fixes nerve-tissue in a 1 per cent. solution of sublimate in 40 per cent. acetone, and washes out through increasingly concentrated grades of acetone.

72. Phenol Solution. PAPPENHEIM (*Arch. Path. Anat.*, clvii, 1899, p. 23) shakes up carbolic acid with aqueous sublimate solution and filters.

73. Ciaccio (*Arch. Ital. Anat. Embr.*, vi, 1907, p. 486) has an irrational mixture of sublimate, iodine, and formol.

74. Mercuro-nitric Mixtures. FRENZEL (*Arch. mik. Anat.*, xxvi, 1885, p. 232) recommends a half-saturated solution of sublimate in 80 per cent. alcohol, to which is added nitric acid in the proportion of 1 drop to 1 or 2 c.c. Objects of the size of a pea to be fixed in it for five or ten minutes, then hardened in the same sublimate alcohol without the acid, and finally in 90 per cent. alcohol. It is said that the nitric acid renders after-treatment with iodine unnecessary.

GILSON'S **Mixture** (GILSON, *in litt.*, 1895).

Nitric acid of sp. gr. 1·456, or 80 per cent., nearly)	15 c.c.
Glacial acetic acid	4 ,,
Corrosive sublimate	20 grm.
60 per cent. alcohol	100 c.c.
Distilled water	880 ,,

When required *for marine animals* add a few crystals of iodine, which will prevent the formation of precipitates of sea salts. If in any case the preparations should show a granular precipitate, this may be removed by washing with water containing a little tincture of iodine.

We find that it affords in general a faithful and delicate fixation, and gives to tissues an excellent consistency. Objects may remain in it for a considerable time without hurt. It has a high degree of penetration. A treatment for a few days with it will serve to remove the albumen from the ova of Batrachians. This liquid *may be recommended to beginners*, as it is very easy to work with. For some objects, the proportion of sublimate may be increased with advantage.

KOSTANECKI and SIEDLECKI (*Arch. mik. Anat.*, cxliii, 1896, p. 181) take a mixture of saturated sublimate solution and 3 per cent. nitric acid in equal parts, or a mixture of equal parts of sublimate solution, 3 per cent. nitric acid, and absolute alcohol, fix for twenty-four hours, and wash out in iodine-alcohol.

PETRUNKEWITSCH (*Zool. Jahrb. Abth. Morph.*, xiv, 1901, p. 576) takes water 300, absolute alcohol 200, glacial acetic acid 90, nitric acid 10, and sublimate to saturation. Both this and Gilson's have been much used lately.

75. Picro-sublimate Mixtures. RABL'S (*Zeit. wiss. Mik.*, xi, 1894, p. 165). Sublimate, saturated solution in water, 1 vol.; a similar solution of picric acid, 1 vol.; distilled water, 2 vols. Embryos may be left in it for twelve hours, washed for two hours in water, and brought into weak alcohol.

O. VOM RATH (*Anat. Anz.*, xi, 1895, p. 268) takes cold saturated solution of picric acid, 1 part; hot saturated solution of sublimate. 1 part; glacial acetic acid, ½ to 1 per cent. Also the same with the addition of 10 per cent. of 2 per cent. osmic acid solution.

76. Osmio-sublimate Mixtures. MANN'S (*Zeit. wiss. Mik.*, xi, 1894, p. 481) consists of a freshly prepared mixture of equal parts of 1 per cent. osmic acid solution and saturated solution of sublimate in normal salt solution. This solution has been much used in recent years for fixation before osmication and is a splendid fixative (see §§ 710 *et seq.*).

77. Chromo-sublimate. LO BIANCO (*Mitth. Zool. Stat. Neapel*, ix, 3, 1890, p. 443). Concentrated sublimate solution, 100 parts; 1 per cent. chromic acid, 50 parts.

MANN (*Verh. Anat. Ges.*, 12, 1898, p. 39) takes for nerve-cells equal parts of 5 per cent. sublimate and 5 per cent. chromic acid.

78. Sublimate and Bichromate. ZENKER's **Mixture** (*Münchener med. Wochenschr.*, xxiv., 1894, p. 534; quoted from MERCIER, *Zeit. wiss. Mik.*, xi, 4, 1894, p. 471). Five per cent. of sublimate and 5 per cent. of glacial acetic acid dissolved in solution of MÜLLER. Fix for several hours or overnight, wash out with water, treat the tissues in bulk, or the sections with alcohol containing tincture of iodine. Refer to § 68.

See also RETTERER, *Jour. Anat. Phys.*, xxxiii, 1897, p. 463, and xxxvii, 1901, p. 480.

If the objects are allowed to remain too long in the fluid there may be formed precipitates, which it is very difficult to remove. SPULER (*Encycl. mik. Technik.*, 1st ed., p. 1280) says that they may be avoided by removing the objects as soon as penetrated, and completing the hardening in liquid of MÜLLER. We recommend this method.

HELLY (*Zeit. wiss. Mik.*, xx, 1904, p. 413) omits the acetic acid and adds, immediately before use, 5 per cent. of formol. This is a splendid fixative for vertebrate material. Fix overnight, wash out in running water for several hours. See footnote to § 698.

MAXIMOW (*ib.*, xxvi, 1909, p. 179) adds 10 per cent. of formol and sometimes 10 per cent. of osmic acid of 2 per cent. (fix in the dark).

FOÀ (*Quart. Journ. Mic. Sci.*, 1895, p. 287) takes equal parts of saturated solution of sublimate in normal salt solution, and of liquid of Müller, or 5 per cent. solution of bichromate.

HOYER (*Arch. mikr. Anat.*, liv, 1899, p. 97) takes 1 part 5 per cent. sublimate and 2 of 3 per cent. bichromate.

KOHN (*ib.*, lxx, 1907, p. 273) takes 5 parts 5 per cent. sublimate, 15 parts 3½ per cent. bichromate, and 1 part acetic acid.

79. Sublamin (Ethylendiamin Sulphate of Mercury) is recommended in 5 per cent. solution by KLINGMÜLLER and VEIEL (*Zeit. wiss. Mikr.*, xxi, 1904, p. 58).

80. Platinum Chloride. The substance used and intended by the authors who have recommended this reagent is not the true platinic chloride, or tetrachloride, $PtCl_4$, but the compound H_2PtCl_6, that is, platinochloric, or hydro-chloro-platinic acid, by custom called platinum chloride. It occurs as brown-red crystals, easily soluble in water and very deliquescent. For this reason it had better be stocked in the form of a 10 per cent. solution, kept in the dark (weak solutions—0·5 per cent.—may be kept in the light).

It appears that some authors have stated that they were using platinous chloride, $PtCl_2$, but that is not possible, as this salt is not soluble in water.

RABL (*Morph. Jahrb.*, x, 1884, p. 216) employed an aqueous solution of 1 : 300. The objects remained in it for twenty-four hours, and were then washed out with water. Well-washed preparations give good chromatin stains with the "basic" tar colours; but we find, as do others, that plasma-staining with the "acid" colours is rendered extremely difficult. It causes a certain shrinkage of chromatin.

It is now almost always employed in the form of mixtures. For

these see §§ 50, 51, 54, as well as the mixtures given under "Picric Acid" and "Formol."

81. Rabl (*Zeit. wiss. Mikr.*, xi, 1894, p. 165) takes for embryos of vertebrates, and also for other objects, 1 vol. of 1 per cent. platinum chloride, 1 of saturated sublimate, and 2 of water.

Lenhossek (*Arch. mikr. Anat.*, li, 1898, p. 220) takes 20 parts of 1 per cent. platinum chloride, 20 of 5 per cent. sublimate, and 1 of acetic acid.

82. **Palladium Chloride** (Schulze, *Arch. mik. Anat.*, iii, 1867, p. 477). Used by Schulze as a hardening agent in a 1 : 800 solution, acidified with hydrochloric acid.

Cattaneo has used it in solutions of 1 : 300, 1 : 600, or 1 : 800 strength, for from one to two minutes, for Infusoria.

Frenkel (*Anat. Anz.*, viii, 1893, p. 538) recommends for connective tissue a mixture of 15 parts 1 per cent. palladium chloride, 5 parts 2 per cent. osmic acid, and a few drops of acetic acid.

83. **Iridium Chloride** (Eisen, *Zeit. wiss. Mik.*, xiv, 1897, p. 195). Solution of $\frac{1}{2}$ or $\frac{1}{5}$ per cent., acidified with 1 per cent. of glacial acetic acid.

With the ovotestis of the snail, Lee has obtained about the worst fixation he has ever seen, but with the testis of *Triton* much better results.

84. **Osmium Chloride** (Eisen, *Journ. of Morph.*, xvii, 1900). Solution of $\frac{1}{2}$ to $\frac{1}{10}$ per cent. From specimens we have seen we should say it is useless.

85. **Perchloride of Iron** (Fol, *Zeit. wiss. Zool.*, xxxviii, 1883, p. 491, and *Lehrb. d. vergl. mik. Anat.*, p. 102). Fol recommends 1 vol. of *Tinct. Ferri Perchlor.* B.P. diluted with 5 to 10 vols. of 70 per cent. alcohol.

The tincture diluted with 3 to 4 vols. of either alcohol or water has been recommended for fixing medullated nerve by Platner (*Zeit. wiss. Mik.*, vi, 1889, p. 187).

86. **Iron Alum.** Strong (*Journ. Comp. Neur.*, xiii, 1903, p. 296) fixes (and decalcifies) heads of young *Acanthias* in 9 parts of 5 per cent. solution of iron alum with 1 of formol, for about two weeks.

87. **Chloride of Zinc** is sometimes used for hardening brain (see Part II). Gilson (*La Cellule*, vi, 1890, p. 122) has used it as a fixative for the silk glands of Lepidoptera, as follows :

Glacial acetic acid	5 c.c.
Nitric acid (80 per cent. nearly)	5 ,,
Alcohol of 80 per cent.	100 ,,
Distilled water	300 ,,
Dry chloride of zinc	20 grm.

88. **Iodine.** Kent (*Manual of the Infusoria*, 1881, p. 114) uses it for fixing Infusoria. Prepare a saturated solution of potassium iodide in distilled water, saturate this solution with iodine, filter, and dilute to a brown-sherry colour. A very small portion only of the fluid is to be added to that containing the Infusoria.

Or you may use Lugol's solution :

Water	100 parts.
Iodide of potassium	6 ,,
Iodine	4 ,,

Or for small marine animals, a solution of iodine in sea-water.

Personally we have found it very useful for the examination of *spermatozoa*. See also under Goodrich's Iodine-Bouin method.

Very small objects may be instantaneously fixed by means of **vapour of Iodine.** Crystals of iodine may be heated in a test-tube till the vapours are given off; then on inclining the tube the heavy vapours may be made to flow over the objects arranged on a slide. The slide should then be warmed to about 40° C. for one to three minutes in order to evaporate the iodine from the objects, which may then be mounted or otherwise treated as desired (OVERTON, *Zeit. wiss. Mik.*, vii, 1890, p. 14).

ORGANIC ACIDS AND OTHER AGENTS

89. Acetic Acid. A substance most injurious to the finer elements of the cytoplasm; in some cases it is indicated for a study of the nuclear elements. Flemming, who has made a special investigation of its action on *nuclei*, finds (*Zellsubstanz*, etc., p. 380) that the best strength is from 0·2 to 1 per cent. Strengths of 5 per cent. and more bring out the nuclear structure clearly at first, but after a time cause them to swell and become pale, which is not the case with the weaker strengths (*ibid.*, p. 103). The *strong* acid is, however, a valuable fixative of certain objects, which it kills with the utmost rapidity, *and leaves fixed in a state of extension.*

The *modus operandi* of VAN BENEDEN is as follows:—Pour glacial acetic acid in liberal quantity over the organisms, leave them until they are penetrated by it—which should be in five or six minutes, as the strong acid is a highly penetrating reagent—and wash out in frequent changes of alcohol of gradually increasing strength. Some persons begin with 30 per cent. alcohol, but this appears to us rather weak, and we think 70 per cent. or at least 50 per cent. should be preferred.

Other energetic reagents may be combined with the glacial acetic acid if desired. Dr. LINDSAY JOHNSON (*in litt.*) has found that one of the best fixatives for retina is a mixture of equal parts glacial acetic acid and 2 per cent. osmic acid. S. LO BIANCO adds to his "concentrated" (49 per cent.) acid one-tenth of a 1 per cent. solution of chromic acid. He finds that even this small proportion of chromic acid serves to counteract in a marked degree the softening action of the acetic acid.

Acetic acid, used *alone*, is only a fixative for *a limited time.* If its action be prolonged, it becomes a *swelling* agent. Its function in mixtures is, besides that of *killing*, the valuable one of counteracting the shrinking action of the ingredients with which it is combined, and by its swelling action enhancing the *penetration* of the mixture; whilst by clarifying tissues it adds to the optical differentiation of their elements.

The proportions in which it should enter into mixtures in general seem to us to be from 0·5 per cent. to 5 per cent. of the glacial acid; higher strengths, such as 25 per cent. to 100 per cent., being only indicated in cases in which the highest possible penetration is the chief consideration.

Throughout this work, wherever acetic acid is mentioned, it is the *glacial* acid that is meant unless the contrary is stated.

All liquids containing a large proportion of this acid (*e.g.* §§ 55, 90) should only be allowed to act for a *very short time.*

90. Acetic Alcohol (CARNOY, *La Cellule*, iii, 1886, p. 6; and *ibid.*, 1887, p. 276; v. BENEDEN et NEYT, *Bull. Ac. Sci. Belg.*,

xiv, 1887, p. 218; ZACHARIAS, *Anat. Anz.*, iii, 1888, pp. 24—27; v. GEHUCHTEN, *ibid.*, 8, p. 227). CARNOY has given two formulæ for this important reagent. The first is—

Glacial acetic acid	1 part.
Absolute alcohol.	3 parts.

The second is—

Glacial acetic acid	1 part.
Absolute alcohol	6 parts.
Chloroform	3 ,,

The addition of chloroform is said to render the action of the mixture more rapid.

V. BENEDEN and NEYT take equal volumes of glacial acid and absolute alcohol.

ZACHARIAS takes—

Glacial acetic acid	1 part.
Absolute alcohol	4 parts.
Osmic acid	a few drops.

Acetic alcohol is one of the most penetrating and quickly acting fixatives known. It preserves both nuclei and cytoplasm, and admits of staining in any way that may be preferred. It was employed by all of the authors quoted for the ova of *Ascaris*—proverbially one of the most difficult objects to fix—but we have found that it is applicable to many other objects. Wash out with 90 per cent. alcohol, and avoid aqueous liquids as far as possible in the after-treatment.

91. Acetic Alcohol with Sublimate. CARNOY and LEBRUN (*La Cellule*, xiii, 1, 1887, p. 68, due to GILSON).

Absolute alcohol	1 vol.
Glacial acetic acid	1 ,,
Chloroform	1 ,,
Sublimate to saturation.	

(The mixture does not keep long, forming ethyl acetate, which precipitates.)

Isolated ova of *Ascaris,* even though furnished with a shell, are fixed in twenty-five to thirty seconds. Entire oviducts take about ten minutes. The liquid is therefore one of the most penetrating and rapidly acting of any.

Wash out with alcohol until all traces of odour or the acetic acid have disappeared (Lee washes out with alcohol containing tincture of iodine). He considers this a very fine reagent.

For Ohlmacher's mixture see § 70.

Murray's Rapid Dehydration Carnoy Method. J. A. MURRAY has suggested using Carnoy's first formula for rapid imbedding. Fix in

the desired method (Bouin formol), transfer to Carnoy, then absolute alcohol.

G. S. Sansom's **Carnoy Modification.**

Absolute alcohol	65 c.c.
Glac. acetic acid	5 ,,
Chloroform	30 ,,

Corr. subl. to saturation. Leave ten minutes to half an hour; wash in iodine absolute, then absolute. (Personal communication.)

Eminently suitable for study of vertebrate material. We have seen some really brilliant results obtained by the use of this fluid (§ 807).

92. **Trichlor-acetic Acid** (Holmgren, *Anat. Hefte*, xviii, 1901, H. 2). This substance is one of the best decalcifying fluids known (§ 601). But swelling of collagen fibres takes place if the fixed material is put in water afterwards. Trichlor-acetic has been used in a number of recent histological fixatives. Use a 4 or 5 per cent. solution in water. Fix (nerve-cells) for eight to twenty-four hours, wash out with alcohol. See also Heidenhain, *Zeit. wiss. Mikr.*, xxii, 1905, p. 321, and xxv, 1909, p. 405, who makes a mixture of 6 per cent. sublimate solution with 2 per cent. of trichlor-acetic and 1 per cent. of acetic acid, which he calls "Subtriessig."

93. **"Susa" Fixatives.** The so-called "Susa mixture" of Heidenhain is as follows: sublimate 4·5 grm., common salt 0·5 grm., distilled water 80 c.c., trichlor-acetic acid 2 grm., acetic acid 4 c.c., formol 20 c.c. Fix one to twenty-four hours, transfer to 90 per cent. alcohol. Ludford informs us that the best way for making it is as follows: stock solution: sublimate 4·5 grm., salt 0·5 grm., water 80 c.c. To make up 10 c.c. of fixative take 8 c.c. of stock and add to it glacial acetic 0·4 c.c., formol 2 c.c., trichlor-acetic 0·2 grm.

The amount of sublimate precipitated in the tissues is slight, and it is usually unnecessary to employ any methods for removing it.

Romeis "Susa" Mixture. Saturated sublimate water 25 c.c., 5 per cent. trichlor-acetic 20 c.c., formol 5 c.c. Fix small pieces one to two hours, larger ones up to twenty-four hours. Transfer to 80 to 90 per cent. alcohol. Recommended for amphibian larvæ (*Zeit. f. Ges. Exper. Mediz.*, Bd. 6).

Note. Trichlor-acetic acid swells collogen if the blocks are taken to water after fixation. Wash out always in 90 to 96 per cent. alcohol.

94. **Trichlor-acetic Fluid for Batrachia** (Champy, *Arch. d. Zool. Expér. et Gén.*, t. lii, 1913).

Carbolic acid cryst. in sat. aq. sol. .	15 parts.
Formol, 40 per cent. . . .	4 ,,
Trichlor-acetic acid, 20 per cent. .	1·5 ,,

Outside of tissue often bad, inner parts better.

95. Chloride and Acetate of Copper (*Ripart et Petit's Liquid*, CARNOY, *La Biologie Cellulaire*, p. 94).

Camphor water (not saturated) .	75	grm.
Distilled water	75	,,
Crystallised acetic acid . .	1	,,
Acetate of copper . . .	0·30	,,
Chloride of copper . . .	0·30	,,

This is a very moderate and delicate fixative, extremely useful for objects that are to be studied in *as fresh a state as possible* in aqueous media. Objects fixed in it stain instantaneously and perfectly with methyl green. Osmic acid may be added to the liquid to increase the fixing action. For *cytological researches* a valuable medium.

96. Nitrate of Copper (GILSON, from GELDERD, *La Cellule*, xxv, 1909, p. 12). Nitrate of copper 200 c.c. sat. sol., formol 500 c.c., sea-water 200 c.c. Seven parts of this solution to be diluted with 100 of sea-water. For Crustacea.

97. Acetate of Uranium (SCHENK, *Mitth. Embryol. Inst. Wien*, 1882, p. 95 ; cf. GILSON, *La Cellule*, i, 1885, p. 141) has a mild fixing action, and a high degree of penetration, and may be combined with methyl green.

FRIEDENTHAL (*Sitzb. Ges. Nat. Freunde Berlin*, 1907, p. 209) recommends equal parts of saturated solution of the acetate and trichlor-acetic acid of 50 per cent.

98. Picric Acid. Picric acid in aqueous solution should be employed in the form of a *strong* solution whenever it is desired to make sections or other preparations of tissues with the elements *in situ*, as weak solutions macerate; but for dissociation preparations or the fixation of isolated cells, weak solutions may be taken. Flemming found that the fixation of nuclear figures is equally good with strong or weak solutions. The saturated solution is the one most employed. (One part of picric acid dissolves in about 86 parts of water at 15° C. ; in hot water it is very much more soluble.) Objects should remain in it for from a few seconds to twenty-four hours, according to their size. For Infusoria one to at most two minutes will suffice, whilst objects of a thickness of several millimetres require several hours.

Picric acid should *always be washed out with alcohol*, that of 70 per cent. being mostly indicated. Staining is better performed by means of alcoholic solutions, or if with aqueous, then with such as are themselves weak hardening agents, such as hæmalum, carmalum, methyl green.

Washing out is facilitated by heat, the extraction being about twice as rapid at 40° C. as at the normal temperature (FOL).

It has been found by JELINEK (*Zeit. wiss Mik.*, xi, 1894, p. 242) that the extraction is greatly quickened by the addition of a base to the wash-alcohol. He recommends carbonate of lithium. A few drops of a saturated solution of the salt in water are added to the alcohol ; a precipitate is formed. The objects are put into the turbid alcohol, which becomes clear and yellow in proportion as the picric is extracted. Further quantities of carbonate are

added from time to time until the colour has been entirely extracted.

Tissues fixed in picric acid can be perfectly stained in any stain. It is seldom necessary to remove the picric acid by washing out before staining. Paracarmine, Boraxcarmine, or Hæmacalcium may be recommended for entire objects.

The most important property of picric acid is its great penetration. This renders it peculiarly suitable for the preparation of chitinous structures.

99. Picric Alcohol (GAGE, *Proc. Amer. Soc. Micr.*, 1890, p. 120). Alcohol (95 per cent.), 250 parts ; water, 250 parts ; picric acid, 1 part.

100. Picro-acetic Acid. BOVERI (*Zellenstudien*, 1, 1887, p. 11) dilutes a concentrated aqueous solution of picric acid with two volumes of water and adds 1 per cent. of acetic acid. According to Lee's experience, the results are miserable.

ZIMMER's mixture (from DEEGENER, *Zool. Jahrb., Abth. Morph.*, xxvii, 1909, p. 634). Saturated aqueous solution of picric acid, 10 parts ; absolute alcohol, 9 ; acetic acid, 1.

101. Picro-sulphuric Acid (KLEINENBERG, *Quart. Journ. Mic. Sic.*, April, 1879, p. 208 ; MAYER, *Mitth. Zool. Stat. Neapel*, ii, 1880, p. 2). MAYER takes distilled water, 100 vols. ; sulphuric acid, 2 vols. ; picric acid, as much as will dissolve.

Liquid of KLEINENBERG is made by diluting the concentrated picro-sulphuric acid prepared as above with three times its volume of water.

Lee holds that the concentrated solution is generally preferable. *This particularly applies to marine organisms.*

Wash out with successive alcohols, beginning with 70 per cent., never with water.

Warm alcohol extracts the acid much more quickly than cold, without which *weeks* may be required to fully remove the acid from chitinous structures.

This liquid may still be useful for Arthropoda, on account of its great power of penetrating chitin ; and for some embryological purposes. For a fuller account see *early editions*.

102. Picro-nitric Acid (MAYER, *Mitth. Zool. Stat. Neapel*, 1881, p. 5)—

Water	100 vols.
Nitric acid (of 25 per cent. N_2O_5) . .	5 ,,
Picric acid, as much as will dissolve.	

Properties of this fluid similar to those of picro-sulphuric acid, with the advantages of avoiding the formation of gypsum crystals, and the disadvantage that it is much more difficult to soak out of the tissues. Mayer states that with eggs containing a large amount of yolk material, like those of *Palinurus*, it gives better results than nitric, picric, or picro-sulphuric acid. Lee considers it distinctly superior to picro-sulphuric for most things. See Hill's fluid, § 803, which gives superior results.

103. Picro-hydrochloric Acid (MAYER, *ibid.*).

Water	100 vols.
Hydrochloric acid (of 25 per cent. HCl) .	8 ,,
Picric acid, as much as will dissolve.	

104. Picro-chromic Acid (Fol, *Lehrb.*, p. 100).

Picric acid, sol. sat. in water . .	10 vols.
1 per cent. chromic acid solution . .	25 „
Water	65 „

We have seen Fol's formula, with the addition of a trace of acetic acid, quoted as "liquid of Haensel."

Lo Bianco takes equal parts of picro-sulphuric acid and chromic acid of 1 per cent.

Rawitz (*Leitfaden*, 1895, p. 24) takes 1 part of picro-nitric acid, and 4 parts 1 per cent. chromic acid. Wash out in 70 per cent. alcohol.

105. Picro-osmic Acid. Flemming (*Zells. Kern u. Zellth.*, p. 381) has experimented with mixtures made by substituting picric for chromic acid in the chromo-osmic mixtures (§ 47), and finds the results identical, so far as regards the fixation of nuclei. The fixation of cytoplasm is in Lee's preparations decidedly inferior.

O. vom Rath (*Anat. Anz.*, xi, 1895, p. 289) adds to 200 c.c. of saturated aqueous solution of picric acid, 12 c.c. of 2 per cent. solution of osmic acid, and 2 c.c. of glacial acetic acid.

Rawitz (*Leitfaden*, p. 24) takes picro-nitric acid, 6 vols.; 2 per cent. osmic acid, 1 vol. Fix for half to three hours. Transfer direct to 70 per cent. alcohol.

106. Picro-platinic and Picro-platin-osmic Mixtures. O. vom Rath (*loc. cit.*, last §, pp. 282, 285) makes a picro-platinic mixture with 200 c.c. saturated aqueous solution of picric acid, 1 grm. of platinic chloride (dissolved in 10 c.c. of water), and 2 c.c. of glacial acetic acid.

The picro-platin-osmic mixture, which is, in Lee's opinion, much superior, is made by adding to the foregoing 25 c.c. of 2 per cent. osmic acid.

Other Picric Mixtures. See §§ 115 and 117.

OTHER FIXING AND HARDENING AGENTS

107. Ethyl Alcohol. For *fixing* only two grades of alcohol should be employed—very weak, or absolute. Absolute alcohol ranks as a fixing agent because it kills and hardens with such rapidity that structures have hardly time to get deformed in the process; very weak, because it possesses a sufficiently energetic coagulating action and yet contains enough water to have but a feeble dehydrating action. The intermediate grades do not realise these conditions, and therefore should not be employed *alone* for fixing. But they may be very useful in combination with other fixing agents by enhancing their penetrating power; 70 per cent. is a good grade for this purpose.

Table for diluting alcohol (after Gay-Lussac). To use this table, find in the upper horizontal row of figures the percentage of the alcohol that it is desired to dilute, and in the vertical row to the left the percentage of the alcohol it is desired to arrive at. Then follow out the vertical and horizontal rows headed respectively by these figures, and the figure printed at the point of intersection of the two rows will show how many

volumes of water must be taken to reduce *one hundred volumes* of the original alcohol to the required grade.

Weaker grade required.	Original Grade. 90 p. 100.	85 p. 100.	80 p. 100.	75 p. 100.	70 p. 100.	65 p. 100.	60 p. 100.	55 p. 100.	50 p. 100.
p. 100. 85	6·56								
80	13·79	6·83							
75	21·89	14·48	7·20						
70	31·05	23·14	15·35	7·64					
65	41·53	33·03	24·66	16·37	8·15				
60	53·65	44·48	35·44	26·47	17·58	8·76			
55	67·87	57·90	48·07	38·32	28·63	19·02	9·47		
50	84·71	73·90	63·04	52·43	41·73	31·25	20·47	10·35	
45	105·34	93·30	81·38	69·54	57·78	46·09	34·46	22·90	11·41
40	130·80	117·34	104·01	90·76	77·58	64·48	51·43	38·46	25·55
35	163·28	148·01	132·88	117·82	102·84	87·93	73·08	58·31	43·59
30	206·22	188·57	171·05	153·61	136·04	118·94	101·71	84·54	67·45

Table for Diluting Rectified Spirit (circa 96 *per cent.*)

Weaker grade required per cent.	Volume of Rectified Spirit.	Volume of Water.
90	93·5	6·5
80	83·3	16·7
70	72·9	27·1
60	62·5	37·5
50	52·1	47·9
30	31·2	68·8

Alcohol is an easily *oxidisable* substance. Chromic acid, for instance, easily oxidises it, first into aldehyde, and then into acetic acid. It follows that alcohol should not be combined in mixtures with oxidising agents of notable energy. Further, alcohol is a *reducing* agent, and therefore should not be combined with easily reducible substances. These remarks particularly apply to chromic acid. See §§ 43–45.

For *fixing*, alcohol is a very third-class reagent, only to be used *alone* where better ones cannot be conveniently employed,

though it enters as a useful ingredient into many *mixtures*, in which it serves to enhance the power of penetration. For *hardening* it is an important one. 90 to 95 per cent. is the most generally useful strength. Weaker alcohol, down to 70 per cent., is often indicated. Absolute alcohol is seldom advisable. You ought to begin with weak, and proceed gradually to stronger, alcohol. Large quantities of alcohol should be taken. The alcohol should be frequently changed, or the tissue should be suspended near the top of it. Many weeks may be necessary for hardening large specimens. Small pieces of permeable tissue, such as mucous membrane, may be sufficiently hardened in twenty-four hours.

108. Absolute Alcohol. This is sometimes valuable on account of its great penetrating power. Mayer finds that boiling absolute alcohol is often the only means of killing certain Arthropoda rapidly enough to avoid maceration.

It is important to employ for fixing a very large proportion of alcohol. Alum-carmine is a good stain for small specimens so fixed. For preservation, the object should be put into a weaker alcohol, 90 per cent. or less.

As to the supposed superiority of absolute alcohol over ordinary strong alcohol, see last § ; and amongst authors upholding its superiority see besides Ranvier, Mayer (*Mitth. Zool. Stat. Neapel*, ii, 1880, p. 7) ; Brüel (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 569) ; and van Rees (*ibid.*, iii, 1888, p. 10).

Absolute alcohol is a product that it is almost impossible to preserve in use, on account of the rapidity with which it hydrates on exposure to air. Fol recommends that a little quicklime be kept in it. This absorbs part at least of the moisture drawn by it from the air.

Ranvier prepares a sufficiently "absolute" alcohol as follows :—Strong (95 per cent.) alcohol is treated with calcined cupric sulphate, with which it is shaken up and allowed to remain for a day or two. It is then decanted and treated with fresh cupric sulphate, and the operation is repeated until the fresh cupric sulphate no longer becomes conspicuously blue on contact with the alcohol ; or until, on a drop of the alcohol being mixed with a drop of turpentine, no particles of water can be seen in it under the microscope. The cupric sulphate is prepared by calcining common blue vitriol in a porcelain capsule over a flame until it becomes white, and then reducing it to powder (see *Proc. Acad. Nat. Sci. Philad.*, 1884, p. 27 ; *Journ. Roy. Mic. Soc.*, 1884, pp. 322 and 984).

Test for the presence of water (Yvon, *C. R. Acad. Sci.*, 1897, p. 1181). Add coarsely powdered calcium carbide ; the merest trace of water will cause an evolution of acetylene gas, and on agitation the alcohol will become turbid.

109. One-third Alcohol. The grade of weak alcohol that is generally held to be most useful for fixing is one-third alcohol, or Ranvier's Alcohol. It consists *of two parts of water and one part of alcohol of* 90 *per cent.* (and *not* of absolute alcohol). See the *Traité Technique* of Ranvier, p. 241, *et passim.*

Objects may be left for twenty-four hours in this alcohol; not more, unless there be no reason for avoiding *maceration*, which will generally occur after that time. You may conveniently stain with picro-carmine, alum-carmine, or methyl green.

This reagent is a very mild fixative. Its hardening action is so slight that it is not at all indicated for the fixing of objects that are intended to be sectioned. Its chief use is for temporary and dissociation preparations.

110. **Pyridin.** Pyridin has been recommended as a hardening agent (by A. de Souza). It hardens, dehydrates, and clears at the same time. It is said to harden quickly, and to give particularly good results with brain. See *Comptes Rendus de Biologie*, 8 sér., t. iv, 1887, p. 622.

This substance is strongly alkaline, and, either pure or diluted with water, dissolves many albumens and fats. It causes considerable shrinkage of nuclei (not so much of cytoplasm). It is now in much use in certain neuro-fibril stains, see Bielschowsky and Ramón. It is soluble in water and in alcohol. Pure, it will harden and dehydrate small brains in a week.

111. **Acetone** is said to harden very rapidly. It precipitates lipins, and may yet prove an important reagent. Scholz (*Zeit. wiss. Mikr.*, xxii, 1905, p. 415) fixes small objects in warm acetone for half an hour to an hour and brings them direct, or through alcohol and ether, into celloidin.

Similarly Fuss (*Arch. path. Anat.*, clxxxv, 1906, p. 5), using it cold and Lintwarew (*ibid.*, ccvi, 1911, p. 36) for erythrocytes, in which it preserves the hæmoglobin.

112. **Lucidol,** see last edition.

113. **Formaldehyde, Formic Aldehyde, Methyl Aldehyde (Formol, Formalin, Formalose).** Formaldehyde is the chemical name of the gaseous compound HCOH, obtained by the oxidation of methyl-alcohol. "Formol," "Formalin," and "Formalose" are commercial names for the saturated (40 per cent.) solution of this in distilled water. This quickly loses in strength through contact with air, and laboratory solutions rarely contain more than 38 per cent., of formaldehyde.

Much confusion has been caused by indiscriminate use of the terms "formaldehyde" and "formol." The proper way is evidently either to state the strengths of solutions in terms of formaldehyde, and say so; or to say "formol—or formalin—with so many volumes of water." The majority of writers seem to state in terms of *formol.*

Solutions of formaldehyde sometimes decompose partially or entirely, with formation of a white deposit of paraformaldehyde. Fish says that to avoid this the solution should be kept in darkened bottles in the cool, or, according to some, it suffices to add glycerin to them.

The solutions almost always have an acid reaction, due to the presence of formic or other acid. W. R. G. Atkins has investigated

the various methods for the neutralisation of formalin solutions, and advocates the use of borax for this purpose. He adds borax till a good red colour is shown with phenolphthalein, or a slaty blue with thymol blue, when added to the *diluted* formalin.

Atkins states further that formalin neutralised with sodium hydroxide becomes acid on standing (*M. B. A. J.*, 1922).

It was said above that formaldehyde possesses *certain* hardening and preserving qualities. It hardens gelatine, for instance, and certain albuminoids; but others, on the contrary, are not hardened by it, but sometimes even rendered more soluble than they are naturally. For some theoretical considerations concerning its action on tissues, see F. Blum, in *Anat. Anz.*, xi, 1896, p. 718; Benedecenti, in *Arch. Anat. u. Phys. Abth.*, 1897, p. 219; Gerota, in *Intern. Monatsschr. Anat.*, xiii, 1896, p. 108; *Zeit. wiss. Mik.*, xiii, p. 311; Sjöbring in *Anat. Anz.*, xvii, 1900, p. 274; and Blum, in *Encycl. mik. Technik.*, p. 393. It seems to be generally admitted that this action consists in the formation of methylene compounds with the substances of the tissues.

The stock should be diluted with isotonic saline, and not in distilled water, according to Carleton (*Q. J. M. S.*, 1922).

We find that, used *pure*, it is far from a first-class fixative. For it over-fixes and shrinks some things, and swells and vacuolates others. But notwithstanding this it is frequently very convenient on account of its *compatibility* with the most *various stains*. It has a high degree of *penetration*, and is a valuable ingredient in many *mixtures*.

It is a powerful reducing agent, and therefore incompatible *with such reagents as chromic acid or osmic acid and the like, which it very rapidly decomposes.*

For *fixing* Lee finds that a strength of about 4 per cent. (1 vol. formol to 9 of isotonic saline, or to 8 of water if the formol has been long kept) is generally about right; and this is the strength used by most writers. For cytological purposes a fixation of at least two days seems indicated: this applies especially to gonads which are notoriously difficult to preserve in formol. The strengths used in Cajal's and Da Fano's formol silver nitrate Golgi apparatus methods, generally give fine results for tissues other than genital. For these, injection fixation may be indicated. See also § 698. Mayer takes 1 of formol to 8 of sea-water, for marine animals. Few workers use much stronger solutions. Only one (Hoyer, *Anat. Anz.*, ix, 1894, p. 236, Erganzungsheft) seems to have used concentrated solutions. We think this exaggerated, for we have found enormous over-fixation with solutions of 1 to 2 vols. of water. Wash out with alcohol (of 50 per cent. or more), not water.

For *hardening*, the same strengths may be taken. Hardening is more rapid than with alcohol. For prolonged hardening, con-

siderable volumes of liquid should be taken, and the liquid should be renewed from time to time; for the formaldehyde fixes itself on the tissues with which it comes in contact, deserting the solution, which thus becomes progressively weaker. The specimens should be suspended in the liquid or otherwise isolated from contact with the containing vessel. The hardening obtained is gentle and tough, giving an elastic and not a brittle consistency. It varies greatly with different tissues. Mucin is not precipitated and remains transparent. Fat is not dissolved. Micro-organisms retain their specific staining reactions. Formaldehyde is said to harden celloidin as well as gelatin, and to be useful for celloidin-imbedding (Blum, *Anat. Anz.*, xi, 1896, p. 724).

Several of the following mixtures are irrational, becoming reduced more or less quickly, but may give good results all the same.

114. Alcoholic Formol (Lavdowsky, *Anat. Hefte*, iv, 1894, p. 361). Water 40 parts, 95 per cent. alcohol 20, formol 6, acetic acid 1; or water 30, alcohol 15, formol 5, acetic acid 1.

Gulland (*Zeit. wiss. Mikr.*, xvii, 1900, p. 222) takes (for blood) 1 part formol and 9 parts of alcohol.

Bles (*Trans. Roy. Soc. Edinburgh*, xli, 1905, p. 792) takes 7 of formol, 90 of alcohol of 70 per cent., and 3 of acetic acid.

Tellyesniczky (*Encyl. mikr. Techn.*, i, p. 472) takes 5 of formol, 100 of alcohol of 70 per cent., and 5 of acetic acid.

Kahle's Fluid (*Die Paedogenesis der Cecidomyiden*, 1908, Stuttgart):

95 per cent. alcohol	15 parts.
Formol (commercial)	6 ,,
Glacial acetic acid	1 part.
Distilled water	30 parts.

This modification of Lavdowsky's fluid is much used these days.

115. Picro-Formol. P. Bouin (*Phénoménes cytologiques anormaux dans L'Histogenèse*, etc., Nancy, 1897, p. 19) recommends—

Picric acid, saturated aqueous sol. .	75 parts.
Formol	25 ,,
Acetic acid	5 ,,

Wash out with alcohol, first of 50 per cent., then 70 per cent. till the picric acid is mostly removed. We consider this to be for most purposes one of the most valuable fixative yet made known. It is rather a strong fixative, and should not be allowed to act for more than eighteen hours. The penetration is great, the fixation equable, delicate detail well preserved, staining qualities admirable, especially with iron-hematoxylin and Saürefuchsin.

Bouin's Fluid and its Modifications

Picric acid, sat. aq. sol. in c.c. .	75	75	75	75	75	75
Formol, in c.c.	25	25	25	15	15	15
Glacial acetic acid, in c.c. . .	5	5	10	10	10	10
Urea, in grams		2	2	1	1	½
Chromic acid, in grams . .		1·5	1·5		1	

Bouin's original formula.
Allen's modification B-15.
Painter's modification of Bouin-Allen.
Allen's P.F.A. 3.
Commonly known as B-3.
Carothers' fluid for orthopteran chromosomes.

See also " Cytology " sections, §§ 625, 1355.

Moreaux (*Bibl. Anat.*, 1910, p. 265) takes 15 parts formol, 85 of trichlor-acetic acid of 3 per cent., and picric acid to saturation.

116. Picro-platinic Formol (M. and P. Bouin, *Bibl. Anat.*, 1898, f. 2, p. 2).

Platinum chloride, 1 per cent. sol. . .	20 parts.
Picric acid, saturated sol. . . .	20 ,,
Formol	10 ,,
Formic or acetic acid	5 ,,

The mixture does not keep more than a day or two, and it is probably inferior.

Bouin also (*Arch. Biol.*, xvii, 1900, p. 211) simply substitutes formol for the osmic acid in Hermann's mixture, § 51.

117. Sublimate Formol (M. and P. Bouin, *loc. cit.*). A similar mixture, in which sublimate of 1 per cent. is substituted for the platinum chloride.

Another formula of the same authors (*Arch. Biol.*, xvii, 1900, p. 211) is 1 part of formol to 3 of saturated aqueous sublimate. Rinse with water and bring into alcohol of 70 per cent.

Spuler (*Encycl. mik. Technik.*, 1st ed., p. 1280) adds to sublimate of 3 per cent. or more 1 per cent. of glacial acetic acid and 10 per cent. of formol.

Mann (*Verh. Anat. Ges.*, 1898, p. 39) takes for nerve-cells 2½ grm. sublimate, 1 grm. picric acid, 5 c.c. formol, and 100 c.c. water, or (*Methods*, etc., p. 97) for all tissues 2½ grm. sublimate, 20 c.c. formol, and 80 c.c. water.

Branca (*Journ. Anat. et Phys.*, xxxv, 1899, p. 767) adds 10 parts of formol and 1 of acetic acid to 60 parts of saturated solution of picric acid with saturated sublimate.

Nowak (*Anat. Anz.*, xx, 1901, p. 244) takes 30 parts of saturated sublimate, 30 of 1 per cent. chromic acid, 27 of water, 3 of acetic acid and 10 of formalin.

118. Formol-Müller. This is the name given by Orth (*Berl. klin. Wochenschr.*, 1896, No. 13) to a mixture of 1 part of formol with 10 of liquid of Müller (§ 58). It should be freshly made up. Fix for three hours in the incubator at 37° C., or twelve at normal

temperature, wash out with running water. Much used, especially for nervous tissues.

MOELLER (*Zeit. wiss. Zool.*, lxvi, 1899, p. 85) takes 1 vol. of formol, and 4 of 3 per cent. bichromate (for the intestine of mammals).

HELD (*Abk. Sächs. Ges. Wiss.*, xxxi, 1909, p. 196) takes 3 per cent. sol. of bichromate with 4 per cent. of formol and 5 per cent. of acetic acid (for inner ear). See also MOREL and BASSAL, *Journ. Anat. Phys.*, xlv, 1909, p. 632, and HELLY and MAXIMOW formulæ. Look up section on "Mitochondria," especially paragraphs on REGAUD and SCHRIDDE, §§ 698–702.

119. Chromic Acid Formol. LO BIANCO fixes marine animals for half to one hour in 10 parts of 1 per cent. chromic acid with 1 of formol and 9 of sea-water, and passes into graded alcohols.

MARCHOUX (from PÉREZ, *Arch. Zool. Exper.*, v, 1910, p. 11) takes 11 parts 1 per cent. chromic acid, 1 of acetic acid, 4 of water, and 16 of formol (added just before using).

These mixtures are neither so good nor so reliable as Bouin's picro-formol.

120. Copper Formol. NELIS (*Bull. Acad. Sc. Belg.*, 1899 (1900), p. 726) fixes spinal ganglia for twenty-four hours in 1 litre of 7 per cent. formol with 5 c.c. of acetic acid, 20 grm. of cupric sulphate, and sublimate to saturation.

STAPPERS (*La Cellule*, xxv, 1909, p. 356) used (for Sympoda) a mixture of GILSON'S: 100 parts of formol of 5 per cent. with 2 gms. of nitrate of copper.

STRONG (*Journ. Comp. Neur.*, xiii, 1903, p. 296) fixes the head of *Acanthias* by injecting a mixture of equal parts of formol and 5 per cent. solution of bichromate of copper.

121. Nitric Acid Formol. WILHELMI (*Fauna u. Flora Golf. Neapel*, xxxii, 1909, p. 15) fixes Triclads in APÁTHY'S mixture of equal parts of 6 per cent. nitric acid and 6 per cent. formol, and brings them direct into strong alcohol.

122. Acetone Formol. BING and ELLERMANN (*Arch. Anat. Phys., Phys. Abth.*, 1901, p. 260) fix medullated nerves in 9 parts of acetone with 1 of formol.

123. Formic Acid. This substance has been used in the past in a number of fixing fluids, *e.g.* in Dr. Lindsay Johnson's mixture (§ 50) and has been more recently investigated by Professor Mary J. Guthrie, who informs us (*in literis*) that promising results have been obtained with this acid as a substitute for acetic acid.

Dr. Guthrie's tests were made because of the information that has been obtained by the cell physiologists that formic acid penetrates more rapidly and is more toxic than acetic acid. In addition, fatty substances are less soluble in formic acid than in acetic.

The most striking results were obtained with fixation of Planaria by a Zenker's fluid to which 5 parts of formic acid, instead of acetic, was added just before using. The worms were allowed to become extended and straightened on a glass slide in a very small amount of water; the fluid was pipetted on, and a No. 1 cover-slip dropped on. After five minutes the cover-slip was removed and the specimens were transferred to a vial containing the fluid. This method of killing causes no writhing with tearing of the muscles, and produces no blistering of the epidermis. The general histological details are excellent,

especially the "formative cells" of the parenchyma. This fixation can be followed by staining with hæmalum and orange G.

Worms treated with Müller's fluid for four days after twenty-four hours' fixation in the Zenker-formic acid mixture give good results with staining in acid fuchsin by the Kull method. Mitochondria are preserved and stain. The addition of 4 parts of 2 per cent. osmic acid to 12 parts of Zenker's stock plus 1 part of formic acid gives preservation of neutral fat, together with the excellent general fixation, and reduces the precipitation of mercuric chloride crystals in the fluid.

The use of formic acid gives better results with the cold fluid than are to be obtained with a hot acetic mixture. In comparison with a Zenker-formalin fluid, such as Helly's, the results are superior.

Dr. Guthrie has also used a strong Flemming's fluid with formic acid (15 parts 1 per cent. chromic acid and 4 parts 2 per cent. osmic acid with 1 part formic acid). Fixation for three days was followed by twenty-four hours in pyroligneous acid and 1 per cent. chromic acid (1 : 2), and twenty-four hours in 3 per cent. potassium bichromate. This gives good histological features and preserves both neutral fat and mitochondria. It can be followed, with splendid results, by staining in crystal violet according to Benda's alizarin method. The crystal violet is a 3 per cent. solution in 95 per cent. alcohol in an equal volume of anilin water.

CHAPTER VI

DEHYDRATION

124. Dehydration is still almost universally carried out with ethyl alcohol, and the other substitutes have made little headway since their introduction into biological laboratories. Ethyl alcohol is supplied as absolute alcohol, or rectified commercial spirit which is about 96 or 97 per cent. The tissues must be dehydrated fairly thoroughly before adding the oil which is the solvent for paraffin wax, or the ether celloidin alcohol mixture for celloidin-imbedding. In recent years various substitutes have been suggested, which dehydrate, and will at the same time dissolve paraffin wax. Thus the so-called de-alcoholisation-stage (xylol, carbon bisulphide) is dispensed with, and the imbedding process hastened.

There is a number of substances which have been developed in connection with the aeroplane " dope " and lacquer industry which have remarkable solvent powers, as, for instance, dioxan (diethylene oxide), and cellosolve (ethylene glycol mono-ethyl ether), and which have been recently proposed for microtomy. Some of these, especially the amyl derivatives, have a physiological action, and should not be used in small or ill-ventilated rooms unless it is certain that they are harmless.

It is generally believed that the dehydration of tissues should be carried out in graded strengths. Some workers always begin at 30 per cent., bringing the material through 50, 70, 90, to absolute alcohol, often a day and night in each strength. Others simply drop the fixed material, after washing, into 90 to 95 per cent. alcohol, and then into absolute alcohol. This may be quite satisfactory for rough histological materials, but is not recommended for delicate objects, such as embryos or insect larvæ. See J. A. Murray's method, § 91.

A table is given on p. 54, which gives figures for diluting 96 per cent. alcohol in order to get the lower strengths.

The dehydration with alcohols is carried out in corked, or glass-stoppered phials, or in Stender dishes with ground or ordinary glass tops. In damp climates the corked bottles are necessary with the higher strengths, but in dry climates the Stender dish method is quite good enough. The periods of immersion of pieces in the various grades of alcohol obviously vary with the size and penetrability of the materials being used. But this is not all—it

is certain that alcohol has a hardening effect, which is, up to a point, of very considerable importance. Thus, while a frog embryo can be dehydrated in less than two hours in the various strengths, the end result is not so good as when the process is lengthened to a day or to forty-eight hours. This hardening effect, in the case of some objects, like insects, is inimical to the best results, and some workers use other varieties of alcohols (§ 127) especially for the higher strengths which harden most. Thus it may be necessary when using ethyl alcohol, to shorten periods in the 90 and 100 per cent. strengths as much as possible consistent with proper dehydration. In § 1240, are some notes on dehydrating plant material.

125. Other Methods of Dehydrating. In the following paragraphs are given a few of the more recently tried methods. It should be noted that for general purposes none of these techniques has the general advantages of ethyl alcohol and xylol or carbon bisulphide, in cheapness and reliability; but for entomological and botanical work, such methods as the amyl alcohol, methylal and dioxan, will have a restricted usefulness. They may be regarded as quite unsuitable for routine work in students' laboratories.

126. Amyl alcohol has been a good deal used since it was suggested by HOLLANDE (*C. R. Soc. de Biol.*, 1918). It is recommended by HARTRIDGE (*Journ. Physiol.*, 1920). This alcohol does not mix with water, but is miscible with 90 per cent. ethyl alcohol, toluol and xylol. Beyond the fact that it is less hydroscopic than ethyl alcohol, it is difficult to see its advantages in routine work. The fact that it does not mix with water is a serious objection to it. It does, however, dissolve paraffin wax, and it is possible to go straight from this alcohol into amyl paraffin mixtures, which in the case of some material, may be very important.

127. N-Butyl alcohol * has been utilised for insects; its use in microtomy was proposed by Mlle. LARBAUD (*C. R. Acad. Sc.*, 1921). It has been especially studied by KARL A. STILES (*Stain Tech.*, 1934), H. M. SMITH (*Turtox News*, 9, 1931) and L. MARGOLENA (*Stain Tech.*, 1932). STILES mentions that he got very good results with insect material. He used Gilson fixed insects which were transferred to 35 per cent. ethyl alcohol for thirty minutes to one hour, then to a mixture of 9 c.c. of 45 per cent. ethyl alcohol + 1 c.c. of butyl alcohol for two hours, then 8 c.c. of 62 per cent. ethyl alcohol + 2 c.c. of butyl alcohol for two hours, then 65 c.c. of 77 per cent. ethyl alcohol + 35 c.c. of butyl for four hours, then 45 c.c. of 90 per cent. ethyl + 55 c.c. of butyl for six hours to days, then 25 c.c. of 90 per cent. ethyl + 75 c.c. butyl for six hours to overnight with one change, finally butyl alcohol alone, two changes at intervals of several hours. Pieces may be stored apparently indefinitely in butyl alcohol.

* See also § 1257.

Imbedding is done by transferring to a mixture of 2 parts paraffin wax to 1 of butyl alcohol (melting-point 56° to 58° C.) in a covered Stender dish in the oven for twelve to twenty-four hours, which is afterwards uncovered until the odour of the butyl alcohol disappears, after which the pieces are transferred to pure paraffin wax. Long periods of infiltration are absolutely necessary, and do not harden either plant, vertebrate, or insect tissues as might be expected.

In defence of this complicated and tedious technique, it may be stated that such insects as aphids cut very nicely, and the end result is probably better than with diaphanol. The times given above are not rigid, insects may be left longer periods in the grades without hurt. It may be noted that only 8·3 grm. of *n*-butyl alcohol are soluble in 100 c.c. of water. The dehydrating power of this alcohol is therefore low.

128. Iso- and normal propyl-alcohols are excellent substitutes for absolute ethyl alcohol and mix in all proportions with water, xylol and cedar oil (W. C. CLOTHIER, *Watson's Microscope Record*, No. 31, 1934.)

129. Ethylene Glycol Mono-Ethyl Ether (Cellosolve) has been suggested as a dehydrating agent by H. F. FROST (*Watson's Microscope Record*, No. 34, 1935). It is expensive, inflammable and rapidly absorbs water from the air. We do not know whether it has any physiological action when breathed in small quantities.

130. Dioxan (Di-ethylene oxide, M.P. 11°, B.P. 101°) has come into use in many laboratories. This liquid is a remarkable solvent. It mixes with water, alcohol, xylol and dissolves balsam and paraffin wax. It is a dangerous substance, and has a marked physiological and cumulative action. It is unsuitable for students' laboratories, and should only be used for special work. Its advantages are not so considerable that they outweigh the fact that it will have an effect on the health of laboratory workers. It has been said that it will preserve intra-vital stains in fixed preparations, and enable stained bodies to be studied in permanent preparations. With neutral red we have not found this to be so, but it is true that very good preparations may be prepared from materials dehydrated and imbedded in dioxan and dioxan wax mixtures. Introduced in 1931 by GRAUPNER and WEISSBERGER (*Zool. Anz.*, Bd. 96) into microtomy, it is much used in chemistry also as a solvent. It is a useful substance for making celluloid cements. Since it has little odour, it is possible to breathe toxic proportions in air, without knowing that one is doing so. It will be particularly dangerous in those small, improperly ventilated rooms so often used for imbedding and section cutting.

With this warning we give the method. From many fixatives it is possible to go straight into dioxan, which is changed three times, and then pass to dioxan and wax—finally pure wax ; thus

with Bouin, Carnoy, Formol, or with fixatives which are washed out in water, as for instance, chrome ones. Corrosive sublimate fixed pieces must go through alcohols as usual to 70 per cent. and iodine, but may then be placed in dioxan. In general, pieces dehydrate in fresh dioxan in twenty-four hours, and should be changed several times. Smaller pieces can be dehydrated very rapidly. We have used dioxan very successfully for imbedding pancreas by bringing the material into equal parts of dioxan and water, pure dioxan, equal parts of dioxan and wax, and so on. We believe this is better than dropping the pieces into pure dioxan. The possibilities of dioxan have not yet been fully investigated.*

After use, the dioxan can be collected and dehydrated by placing a bag of calcium chloride in the bottle. Some workers use a wide-mouthed glass-stoppered jar with a copper gauze layer near the bottom and calcium chloride below the gauze. Pieces to be dehydrated are dropped on to the gauze and recovered with forceps after over-night immersion.

131. Methyl benzoate ($C_6H_5 . COO . CH_3$) B.P., 198·6° C. S.G. 1·0942. Refractive index 1·517. This substance has been used for many years on the Continent, but only recently has it become popular in British and American laboratories. It is said to remove the last traces of water left after dehydrating in ethyl alcohol, and by clearing the blocks or embryos, enables one to see how successfully dehydration has been carried out. Since its refractive index is very close to that of cedarwood oil, it is now used as immersion oil, with the advantage that it need not be wiped off, as it evaporates.

It is used as follows in imbedding. From absolute alcohol the piece of tissue is passed through two changes of pure methyl benzoate, twelve to twenty-four hours in each, according to size of piece. The material will clear beautifully. Then transfer to benzol (two changes for twenty to thirty minutes) and through to a mixture of wax and benzol. (Refer also to § 177.)

Some people go from 96 per cent. ethyl alcohol to methyl benzoate for objects which would harden too much in absolute alcohol. Material does not harden in the benzoate.

PETERFI (*Zeit. f. wiss. Mikr.*, 38) has a method of passing pieces of tissue dehydrated in alcohol into three changes of 1 per cent. celloidin in methyl benzoate, then in benzol, and benzol wax mixture. Methyl benzoate is not a cheap substance, and for most purposes it is sufficient to see that the absolute alcohol is all right.

132. Methylal Method (L. GENEVOIS, J. DUFRENOY, *Sci.*, October, 1935). Methylal or Formal is methylen dimethyl ether $CH_2(O . CH_3)_2$, B.P. 42° C., dissolves 3 parts water. Genevois has suggested a method favourably reported by Dufrenoy. Material is fixed and then brought

* It has recently been suggested to use dioxan as the basis of fixatives just as alcohol is used in Carnoy, see § 672.

into water which must permeate the pieces. It is then transferred to a *mixture* of water and methylal in equal parts, then pure methylal, then methylal dehydrated in anhydrous sodium carbonate; after these baths the pieces are placed in equal parts of methylal and paraffin oil. The dish is warmed up on a water bath, and then transferred to soft melted paraffin, and then finally into the hard paraffin. One hour for each stage is the time given by Dufrenoy. It is claimed that this method does not harden tissues, and its solvent power on cell granules is negligible. Methylal is rather expensive. We have had no success with this method.

CHAPTER VII

DE-ALCOHOLISATION AND CLEARING AGENTS

133. Introduction. *De-alcoholisation agents* are liquids employed for the purpose of getting rid of the alcohol which has been employed for dehydrating tissues (§ 124), and facilitating the penetration of the paraffin used for imbedding, or the balsam or other resinous medium in which preparations are, in most cases, finally mounted. Hence all of them must be capable of expelling alcohol from tissues, and must be at the same time solvents of Canada balsam and the other resinous mounting media. The majority of them are essential oils.

Clearing agents are liquids whose functions it is to make microscopic preparations transparent by penetrating amongst the highly refracting elements of which the tissues are composed, the clearing liquids themselves having an index of refraction superior, or equal, or, at all events, not greatly inferior, to that of the tissues to be cleared Hence all clearing agents are liquids of high index of refraction.

The majority of de-alcoholisation agents being also liquids of high refraction, it follows that they serve at the same time for de-alcoholisation and for clearing; and in consequence it has come about that de-alcoholisation agents are generally spoken of as clearing agents. But that practice is not strictly correct, for not all clearing agents are solvents of the resins, and not all de-alcoholising agents can serve as clearers. We shall, however still in many cases continue to use the term "clearing" to signify "de-alcoholising," for the sake of brevity.

NEELSEN and SCHIEFFERDECKER (*Arch. Anat. Phys.*, 1882, p. 206) examined a large series of ethereal oils (prepared by Schimmel & Co., Leipzig), with the object of finding a not too expensive substance that should combine the properties of clearing quickly alcohol preparations, *not* dissolving out anilin colours, clearing celloidin without dissolving it, and not evaporating too quickly.

Of these, the following three fulfil the conditions: *Cedar-wood, Origanum, Sandal-wood.*

To these should be added the others recommended in the following paragraphs.

See also the paper of JORDAN (*Zeit. wiss. Mik.*, xv, 1898, p. 50) as to the behaviour of some essential oils towards celloidin.

134. The Practice of De-alcoholisation or Clearing. The old plan was to take the object out of the alcohol and float it on the surface of the de-alcoholising or clearing medium in a watch-glass. This plan was faulty, because the alcohol escapes from the surface of the object into the air quicker (in most instances) than the de-alcoholising or clearing agent can get into it; hence the object must shrink. To avoid this cause of shrinkage, the operation is now generally done by the method suggested by Mayer and Giesbrecht, which consists in putting the clearing medium *under* the alcohol containing the object. The objects should not be considered to be perfectly penetrated by the clearing medium until the wavy refraction-lines caused by the mixture of the two liquids at their surface have ceased to form, and they should not be mounted or imbedded until they have first been soaked for some time in a fresh quantity of clearing medium, to remove any alcohol still remaining.

The penetration of all clearing media may be hastened by using them *warm*.

It frequently happens that the essential oil with which objects are being treated in a watch-glass or on a slide becomes cloudy after a short time, and fails to clear the tissues. This is owing to a combination between the essential oil and moisture derived, we think, rather from the air than from the objects themselves. The cloudiness can usually be removed by warming (as pointed out by Hatchett Jackson, *Zool. Anzeig.*, 1889, p. 630), but in certain moist states of the atmosphere it may persist, notwithstanding continued warming. It is for this reason that we advise that clearing be done, whenever possible, in shallow *corked tubes*, under which conditions the phenomenon rarely occurs. In any case, be careful not to breathe on the liquid.

135. Choice of a De-alcoholisation or Clearing Agent. In recent years carbon bisulphide has begun to oust xylol or benzol from many of our laboratories for routine work, and the method of getting the dehydrated pieces between a layer of the oil and the alcohol in a phial (§ 4) is much used, instead of adding benzol or carbon bisulphide little by little to the Stender dish of alcohol. The introduction of dioxan, and the use of alcohols which dissolve paraffin, used in special work, has also to be noted (§§ 124–131). The use of methyl benzoate instead of cedarwood oil, is to be recommended, as the former washes out in benzol much better than the latter in the two-stage clearing used for large pieces and embryos. We advise the beginner to keep on his table the following: Oil of cedar and methyl benzoate, for general use and for preparing objects for imbedding in paraffin; clove oil, for making minute dissections in (§ 8), and for much work with safranin, etc., oil of bergamot, which will clear from 90 per cent. alcohol, and which does not extract coal-tar colours; carbol-xylol and carbolic acid, for rapidly clearing very imperfectly dehydrated objects.

136. Clearing Whole Mounts of Fresh-Water Micro-Fauna. Peter Gray (Watson's *Microscope Record*, No. 37, 1936), remarks: Clove oil is rapid, but makes objects brittle; terpineol is cheaper, safer, pleasanter, but much slower. Beechwood creosote (B.P.) is probably the best all round reagent, it clears imperfectly dehydrated objects gently and effectively, leaving them flexible. Do not use cedarwood oil, xylol or methyl salicylate, as these need more perfect dehydration.

For special clearers for *celloidin sections* see Chapter X.

137. Cedar Oil (Neelsen and Schiefferdecker, *loc. cit.*, § 133). Clears readily tissues in 95 per cent. alcohol *without shrinkage;* does not extract anilin colours. Celloidin sections are cleared in five to six hours.

The observer should be careful as to the quality of the cedar oil he obtains. We have examined the clearing properties of a sample, obtained from a celebrated firm, which *totally* failed to clear absolute alcohol objects after many days.

Cedar oil is *very penetrating*, and for this and other reasons is, in our experience, the very best of all media for preparing objects for paraffin imbedding. We find it to be *less hurtful to cells* than any other medium known to us. Tissues may remain in it for any length of time without hurt. If it should become milky through keeping, filter.

Cedar oil has been largely superseded by methyl benzoate (§ 131).

138. Clove Oil. Samples of clove oil of very different shades of colour are met with in commerce. It is frequently recommended that only the paler sorts should be employed in histology. Doubtless it is, in general, best to use a pale oil, provided it be pure; but it is not always easy to obtain a light-coloured oil that is pure. Clove oil passes very readily from yellow to brown with age, so that in choosing a colourless sample you run great risk of obtaining an *adulterated* sample, for clove oil is one of the most adulterated oils in commerce.

Clove oil does not easily spread itself over the surface of a slide, but has a tendency to form very *convex drops*. This property makes it a very convenient medium for making minute dissections. It also has the property of making tissues that have lain in it for some time very *brittle*. This brittleness is also sometimes very helpful in minute dissections.

These qualities may be counteracted if desired by mixing the clove oil with bergamot oil.

This is one of the most useful of clearers. According to Behrens (*Tabellen*, 3rd ed., 1898, p. 33), it will clear from alcohol of 74 per cent.

It has a high index of refraction, and clears objects *more* than balsam mounting media. It dissolves celloidin (or collodion), and therefore should not be used for clearing sections cut in that medium without special precautions. New clove oil washes out basic tar colours more quickly than old.

139. **Cinnamon (or Cassia) Oil** greatly resembles clove oil, but is in general thinner, and is more highly refractive. An excellent medium, which we particularly recommend.

140. **Oil of Bergamot** (SCHIEFFERDECKER, *Arch. Anat. Phys.*, 1882 [Anat. Abth.], p. 206.) Clears 95 per cent. alcohol preparations and celloidin preparations quickly, and does not extract anilin colours.

Bergamot oil is the least refractive of these essences, having a lower index than even oil of turpentine.

SUCHANNEK (*Zeit. wiss. Mik.*, vii, 1890, p. 158) says that bleached, colourless bergamot oil will not take up much water, whereas a green oil will take up as much as 10 per cent.

VAN DER STRICHT (*Arch. de Boil.*, xii, 1892, p. 741) says that bergamot oil will, with time, dissolve out the fatty granules of certain ova.

141. **Oil of Origanum** (NEELSEN and SCHIEFFERDECKER, *Arch. Anat. Phys.*, 1882, p. 204). Ninety-five per cent. alcohol preparations are cleared quickly, and so are celloidin sections, without solution of the celloidin. Anilin colours are somewhat extracted.

For work with celloidin sections care should be taken to obtain *Ol. Origani Cretici* ("Spanisches Hopfenöl"), not *Ol. Orig. Gallici* (v. GIESON ; see *Zeit. wiss. Mik.*, iv, 1887, p. 482). Specimens of origanum oil vary greatly in their action on celloidin sections.

SQUIRE, in the *Methods and Formulæ*, etc., p. 81, says that origanum oil (meaning the commercial product) is nothing but oil of white thyme more or less adulterated (see next §), and that the product sold as Ol. Origani Cretici is probably oil of marjoram.

142. **Oil of Thyme.** FISH (*Proc. Amer. Mic. Soc.*, 1893 ; *Zeit. wiss. Mik.*, xi, p. 503), following BUMPUS, says that for most of the purposes for which origanum oil has been recommended, oil of thyme will do just as well if not better. The *red* oil is just as sufficient as the *white* for clearing.

Schimmel & Co., in their Report of October, 1895, p. 69, state that in France white oil of thyme is adulterated with oil of turpentine to the extent of as much as 50 per cent.

143. **Oil of Gaultheria.** Used by UNNA (*Monatschr. prakt. Derm.*, *Ergänzungsh.*, 1885, p. 53) for thinning balsam. The artificial oil, **methyl salicylate**, is recommended by GUÉGUEN (*Comp. Rend. Soc. Biol.*, v, 1898, p. 285) both as a de-alcoholisation and clearing agent and as a solvent of paraffin. The refractive index is 1·53. It is, unfortunately, easily spoilt by water.

144. **Sandal-wood Oil** (NEELSEN and SCHIEFFERDECKER, *loc. cit.*). Very useful, but its high price is prohibitive.

145. **Oil of Cajeput.** See last edition.

146. **Oil of Turpentine.** Generally used for dissolving out the paraffin from sections ; but many other reagents, such as xylol and benzol, are preferable for this purpose. If used for alcohol objects, it causes considerable shrinkage, and *alters the structure of cells* more than any other clearing agent known to me. Turpentine has the lowest index of refraction of all the usual clearing agents except bergamot oil ; it clears objects *less* than balsam. (See under "Cytology," § 710.)

147. **Terpineol** (liquid, from Schimmel & Co.) is recommended by MAYER, *Zeit. wiss. Mikr.*, xxvi, 1910, p. 523. Clears from

alcohol of 90 per cent., or even 80 per cent. One part xylol and 4 terpineol has been much used lately.

148. Carbolic Acid. Best used in concentrated solution in alcohol. Clears instantaneously, even very watery preparations. This is a very good medium, but it is better avoided for preparations of soft parts which it is intended to mount in balsam, as they generally shrink by exosmosis when placed in the latter medium. It is, however, a good medium for celloidin sections.

GAGE'S **Mixture** (*Proc. Amer. Soc. Micr.*, 1890, p. 120). Carbolic acid crystals melted, 40 c.c.; oil of turpentine, 60 c.c.

149. Creosote. Much the same properties as carbolic acid. *Beech-wood* creosote is the sort that should be preferred for many purposes—amongst others, for clearing celloidin sections, for which it is a very good medium. Note Gray's remarks (§ 136).

150. Anilin Oil. Common anilin oil will readily clear sections from 70 per cent. alcohol, and with certain precautions (see the paper by SUCHANNEK, *Zeit. wiss. Mikr.*, vii, 1890, p. 156, or the third edition of this book), objects may be cleared from watery media without the intervention of alcohol at all.

In recent years the use of anilin oil, for clearing delicate tissues, *e.g.* mammalian testicular material (§ 629) has become widespread because the higher grades of alcohol may be avoided. *It is to be noted, however, that it should not be used following osmic acid fixation.* Ordinarily, one begins with tissues in 50 or 70 per cent. alcohol and gradually replaces the alcohol with anilin oil. After clearing the anilin oil should be washed out of the tissue by two or three changes of chloroform, or some similar reagent, because it does not mix well with paraffin.

On standing commercial anilin tends to discolour through oxidation, and if exposed to air, it may absorb water. While the change of colour does not spoil it for use, the lighter shades are to be preferred. (Old oil should be redistilled.)

Anilin is chiefly used for clearing celloidin sections. It ought, however, to be soaked out before mounting by something else (chloroform or xylol for instance for some hours), as if not removed it will brown both the tissues and the mounting medium.

151. Chloroform. For clearing tissues before imbedding chloroform has long been a favourite reagent in many laboratories. It is an excellent de-alcoholisation agent, as it will take up a good deal of water, if any be left in the tissue and it seems to render many tissues less brittle than xylol. The one drawback of chloroform is that it does not penetrate well, and its use should therefore be restricted to small objects which are easily penetrable.

152. Xylol, Benzol, Toluol. Too volatile to be recommendable as clearing agents in which it is desired to *examine* specimens, but very useful for clearing small objects.

Xylol is the clearing agent in most common use at present. It will mix with 95 per cent. alcohol, but it is advisable to pass objects through absolute alcohol in order to ensure complete dehydration. Xylol has a tendency to harden all tissues, if they are left in it too long, and yolk ladened tissues are rendered especially hard and brittle. For the latter one should use either some other clearing agent, described below, or some measure for keeping the tissue soft (see § 138).

Both xylol and toluol are liable to become acid if kept too long in partially filled vessels. To remove water from cheap xylol suspend a bag of fused copper sulphate in the jar.

152a. Carbol-xylol. In moist localities it is often difficult to clear alcoholic material with xylol alone and carbol-xylol must be used. (Made by adding anhydrous crystals of pure phenol to xylol until no more will dissolve.) After clearing the object can be rinsed in pure xylol, if desired.

Some workers prefer a mixture of equal parts of xylol and beechwood creosote for clearing.

153. Normal Butyl Alcohol. The use of this reagent as a dehydrating and clearing fluid is relatively new. It was first suggested, apparently by Mlle. LARBAUD (*Comp. Rend. Acad. Sci.*, 172, 1921, p. 1317), but was brought to the fore by ZIRKLE (*Science*, lxxi, 1930, p. 103), who uses and recommends it for woody tissues which are rendered too hard and brittle for cutting after ethyl alcohol-xylol treatment. More recently, it has been found valuable for animal tissues as well, especially for lightly chitinised insects which must be sectioned (STILES, *Stain Tech.*, 9, 1934, p. 97). The advantages claimed for *n*-butyl alcohol are that the hardening effects of higher grades of ethyl alcohol and xylol are avoided by its use, and a long time may be taken for dehydration and clearing without any deleterious effect upon the tissue. On the other hand, it requires four or five days to infiltrate tissue with paraffin by this method, a fact which may restrict its use. For a detailed description of the method see §§ 127 and 1257.

154. Isopropyl Alcohol. BRADBURY (*Science*, lxxiv, 1931, p. 225) has used isopropyl alcohol as a substitute for ethyl alcohol, the main advantage being that the former does not harden tissue as much and makes the sectioning of much material easier. (See also § 128.)

CHAPTER VIII

IMBEDDING METHODS—INTRODUCTION

155. Imbedding Methods. The processes known as Imbedding Methods are employed for a twofold end. Firstly, they enable us to surround an object, too small or too delicate to be firmly held by the fingers or by any instrument, with some plastic substance that will support it on all sides with firmness but without injurious pressure, so that by cutting sections through the composite body thus formed the included object may be cut into sufficiently thin slices without distortion. Secondly, they enable us to fill out with the imbedding mass the natural cavities of the object, so that their lining membranes or other structures contained in them may be duly cut *in situ*; and, further, they enable us not only to surround with the supporting mass each individual organ or part of any organ that may be present in the interior of the object, but also to fill with it each separate cell or other anatomical element, thus giving to the tissues a consistency they could not otherwise possess, and ensuring that in the thin slices cut from the mass all the minutest details of structure will precisely retain their natural position.

These ends are usually attained in one of two ways. Either the object to be imbedded is saturated by soaking with some material that is liquid while warm and solid when cold, which is the principle of the processes here called *Fusion Imbedding Methods;* or the object is saturated with some substance which whilst in solution is sufficiently fluid to penetrate the object to be imbedded, whilst, after the evaporation or removal by other means of its solvent, it acquires and imparts to the imbedded object sufficient firmness for the purpose of cutting. The methods founded on this principle are here called *Evaporation Imbedding Methods*.

In any of these processes the material used for imbedding is technically termed an " imbedding mass."

There are two chief methods of imbedding—the paraffin method and the celloidin or collodion method.

The paraffin method is the one in most use; for it is the *more rapid*, requiring only hours where the celloidin process requires days or weeks; and it is the one which the most readily affords very thin sections. But this only applies to fairly small objects; with objects of much over half an inch in diameter you cannot

easily get with paraffin much thinner sections than you can with celloidin; and if you try to cut in paraffin objects of still greater size, say an inch and upwards, it will frequently happen that you will not get perfect sections at all, blocks of paraffin of this size having a tendency to *split* under the impact of the knife. This defect is, however, much reduced by the employment of a softer paraffin than is usual. In this way Strasser (*Zeit. wiss. Mik.*, ix, 1892, p. 7) has obtained series of frontal sections 30 μ thick through the entire human brain, in paraffin blocks measuring 10×15 cm. And Mayer, with the Tetrander microtome, has obtained series of only 7·5 μ with a surface of $4\frac{1}{2} \times 3$ cm.

For very large objects celloidin is safer, because it does not split, and presents advantages for the manipulation of the sections obtained. For all classes of objects it has the advantages of affording a *transparent* mass (which facilitates orientation of the object) and of producing *less shrinkage* than paraffin (paraffin unavoidably shrinks on cooling to at least 12 per cent.). It is for these two reasons that celloidin is so frequently preferred by embryologists—even for small objects.

Aqueous masses, such as gum or gelatin, may render great service in cases in which it is desired to *avoid dehydrating* tissues, and to apply *chemical tests* to them.

The laboratory worker should note the methyl benzoate (§ **131**), *the dioxan* (§ 130), *the n-butyl alcohol* (§ 127), *and the ceresin wax methods* (§ 177), *as newer techniques which may prove helpful. Attention may be called to* § 157 *for recently used imbedding manipulations.*

156. Imbedding Manipulations. Imbedding in a melted mass, such as paraffin, is performed in one of the following ways. A little tray or box or thimble is made out of paper, some melted mass is poured into it, and the object placed in the midst of it. Or, the paper tray being placed on cork, the object may be fixed in position in it whilst empty by means of pins and the tray filled with melted mass at one pour. The pins are removed when the mass is cold.

In either case, when the mass is cold the paper is removed from it before cutting.

To make **paper trays** proceed as follows. Take a piece of stout paper or thin cardboard, of the shape of the annexed figure (Fig. 1); thin (foreign) post-cards do very well indeed. Fold it along the lines *a a′* and *b b′*, then along *c c′* and *d d′*, taking care to fold always the same way. Then make the folds *A A′*, *B B′*, *C C′*, *D D′*, still folding the same way. To do this you apply *A c* against *A a*, and pinch out the line *A A′*, and so on for the remaining angles. This done, you have an imperfect tray with "dogs' ears" at the angles. To finish it, turn the dogs' ears round against the ends of the box, turn down outside the projecting

flaps that remain, and pinch them down. A well-made post-card tray will last through several imbeddings, and will generally work better after having been used than when new.

Another method of folding the paper (Mayer) is described in the *Grundzüge*, Lee and Mayer, 4th ed., p. 77.

Giesbrecht now makes trays of photographic films, which, being transparent, facilitate orientation under the dissecting microscope.

To make **paper thimbles**, take a good cork, twist a strip of paper several times round it so as to make a projecting collar, and stick

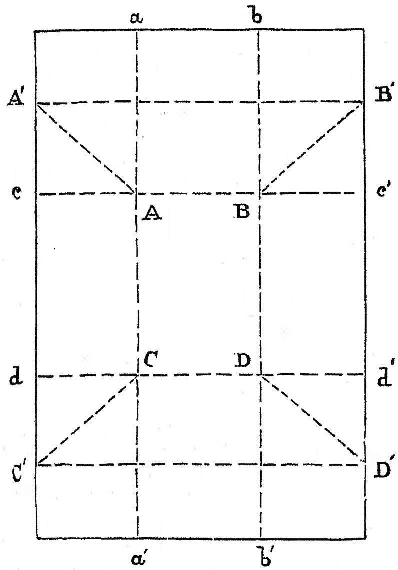


Fig. 1.

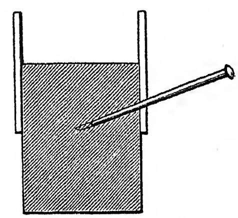


Fig. 2.

a pin through the bottom of the paper into the cork. For work with fluid masses, such as celloidin, the cork may be loaded at the bottom by means of a nail or piece of lead, to prevent it from floating when the whole is thrown into spirit or other liquor for hardening (Fig. 2). Or you may use short lengths of solid lead rod instead of cork.

Leuckhart's **Imbedding Boxes** are made of two pieces of type-metal (Fig. 3). Each of these pieces has the form of a carpenter's " square " with the end of the shorter arm triangularly enlarged outwards. The box is constructed by placing the two pieces together on a plate of glass which has been wetted with glycerin and gently warmed. The area of the box will vary according to the position given to the pieces, but the height can be varied only by using different sets of pieces. Two sets will

be sufficient for most work, one set of 1 cm. in height, and one of 2 cm., each being 8 cm. in length, and 3 in breadth. To make the box paraffin-tight, so that it will hold the melted paraffin long enough in the liquid state to permit of the objects being carefully orientated in it, MAYER (*Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429) first smears the glass plate with glycerin, then arranges the metal "squares," and then fills the box with collodion, which is poured out again immediately. As the ether evaporates, a thin layer of collodion remains behind, which suffices to keep the paraffin from running out. Even without the collodion, the mere cooling of the paraffin by the metal will generally suffice to keep it in long enough for orientation, if it is not in a superheated state when it is poured in.

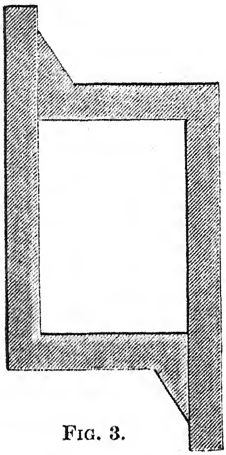


FIG. 3.

In such a collodionised box the paraffin may be kept in a liquid state by warming now and then over a spirit lamp, and small objects be placed in any desired position under the microscope (*Journ. Roy. Mic. Soc.* [N.S.], ii, p. 880).

A lighter form of "squares" made of brass and devised by ANDRES, GIESBRECHT, and MAYER, is described *loc cit.* (See *Journ. Roy. Mic. Soc.*, 1883, p. 913.) A more complicated sort is described by WILSON in *Zeit. wiss. Mik.*, xxvii, 1910, p. 228, for use with imbedded threads to serve as orientation guides. See "Orientation."

FRANKL (*Zeit. wiss. Mik.*, xiii, 1897, p. 438) builds up boxes with rectangular blocks of glass, which may be found convenient, but are more expensive than the metal squares.

SELENKA has described and figured another sort of apparatus having the same object. It consists of a glass tube, through which a stream of warm water may be passed and changed for cold as desired, the object being placed in a depression in the middle of the tube (see *Zool. Anz.*, 1885, p. 419). A simple modification of this apparatus, which any one may make for himself, is described by ANDREWS in *Amer. Natural.*, 1887, p. 101 ; and a more complicated imbedding and orienting box, seldom necessary, is described by JORDAN in *Zeit. wiss. Mik.*, xvi, 1899, p. 32.

To imbed in a **watch-glass** (previously rubbed around with glycerin), the object, saturated with paraffin, is put into a (preferably very concave) watch-glass containing molten paraffin. After this has been solidified by cooling (see next chapter), a block containing the object is cut out of it, and mounted on the object-holder of the microtome (this is, of course, *applicable to other masses*, such as celloidin).

157. Handling and Imbedding Very Small Objects. When dealing with ova of many marine invertebrates, the ovaries of Drosophilæ, and other small objects, much time and valuable material is saved if they are enclosed, in some way, so as to prevent loss during dehydration and imbedding. In this section are brought together some of the devices with which we are familiar, but in several instances we are unable to cite the originator of the method.

After any fixation which requires washing in running water, Painter's method is to place the material in a short piece of glass tubing bent into the form of a J. The diameter of the tubing should be large, say 6 to 10 mm. The tube is placed upright in a small glass dish and water is dropped into the long arm, by a capillary syphon. Should any of the material be washed out of the tube it will be caught by the glass dish in which the tube is placed. This method works well with ovaries of Drosophilæ.

After fixation and washing, small objects can be mixed in a drop or two of white of egg. With a scalpel, the mass is scraped off on to the edge of a slip of paper and the albumen coagulated in 70 per cent. alcohol, or a 5 per cent. solution of corrosive sublimate, or a 2 per cent. solution of chromic acid. The choice will depend on the method of preservation used in the first place. The paper slip, which can be labelled, serves as a convenient handle and need not be detached until the material is actually imbedded.

In America it is a common practice to employ the inner skin from a *Drosophila* pupa as a container for small objects which are to be imbedded and sectioned. Lying just beneath the hard outer pupal case, in *Drosophila* species, there is a thin membrane surrounding the pupa which can be easily removed with needles a day or two before the adult emerges. The skin which surrounds the abdomen and which will usually come off intact, is the part used as a case into which ovaries and like material can be pushed with the aid of a blunt curved needle under a dissecting microscope. Painter has found it desirable to insert into the pupal skin a blunt curved needle and straighten out the wall, immediately after removal from the pupa and then to transfer the skin on the needle to 50 or 70 per cent. alcohol. This causes the skin to harden somewhat and is a convenient method of storing the cases. In use, it is better to place the ovaries in the pupal skin just after washing or while they are in 35 per cent. alcohol, because in higher alcohols they become friable and crush easily. The skin containing the ovaries may now be passed through the higher alcohols, stained with eosin, if desired, and cleared and imbedded in the usual way.

When a considerable amount of material is available it can be drawn into a pipette and placed in the middle of a piece of more or less rectangular shaped membrane, such as the skin shed by many

Amphibia or the amnion from embryos of higher vertebrates. The four corners of the skin can now be gathered up and twisted together with the aid of forceps, and transferred to the higher grades of alcohol which will quickly harden the skin so that it will not untwist. The skin with the contained material can now be dehydrated, stained with eosin if desired, cleared and imbedded in the ordinary way. Painter used this method in Dr. Boveri's laboratory.

A method which Gatenby uses for small objects and which originated probably on the Continent, is to sharpen a rod rather bigger than a lead pencil. Silver foil from cigarette packets is pressed over the end to form a cone. These cones have smooth sides and the objects fall to the bottom. The entire dehydration and imbedding process is carried out in the cone (propped up in a small Stender dish), the liquids being gently sucked out with a pipette. When cooled the tip of the paraffin block containing the objects is re-imbedded in a block of cold wax, in which a hole has been made with a hot wire.

For imbedding *very small objects* in this way certain precautions may be necessary in order not to lose them. SAMTER (*Zeit. wiss. Mik.*, xi, 1894, p. 469) saturates small unstained objects with paraffin that has previously been strongly coloured with alkanna extract, and then imbeds them in pure paraffin. RHUMBLER (*ibid.*, xii, 1895, p. 312, and xiii, 1896, p. 303) stains previously the objects themselves with eosin dissolved in strong alcohol, and removes the stain from the sections with weak alcohol. See also *ibid.*, xiii, p. 200, a paper by SCHYDLOWSKI ; and in *Zeit. wiss. Zool.*, lviii, 1897, p. 144, a process of BORGERT.

BORGERT (*Zeit. wiss. Zool.*, lviii, 1897, p. 144) allows paraffin to solidify in a watch-glass, bores a hole in it, and places the objects in the hole with a little benzol, and puts the whole for a short time into a stove.

A watch-glass provided at the bottom with a groove or trough, in which small objects may be made to collect, is described by LEFÈVRE, *Journ. App. Mic.*, v, 1902, p. 280 (see *Journ. Roy. Mic. Soc.*, 1903, p. 233).

LAUTERBORN (*Zeit. wiss. Zool.*, lix, 1895, p. 170) brings the objects through chloroform into paraffin in a small glass tube, and after cooling breaks the tube and so obtains a cylinder of paraffin with the objects ready for cutting.

HOYER (*Arch. mik. Anat.*, liv, 1899, p. 98) performs all the operations in a glass cylinder (5 cm. long and 7 mm. wide), open at both ends, but having a piece of moist parchment paper tied over one of the openings. It is then not necessary to break the cylinder ; by removing the parchment paper the paraffin can be pushed out of it in the shape of a cylinder containing the objects imbedded at one end of it.

MAYER (*Zeit. wiss. Mikr.*, xxiv, 1907, p. 130) takes the gelatin capsules used by chemists : after cooling in water the gelatin swells and is easily removed.

MEVES (*Arch. mikr. Anat.*, lxxx, Abth. ii, 1912, p. 85) employs wedge-shaped capsules made by G. Pohl, Schönbaum, Bez, Dantzig.

CHAPTER IX

IMBEDDING METHODS—PARAFFIN AND OTHER FUSION MASSES

158. Saturation with a Solvent. The first stage of the paraffin method consists in the saturation of the object with some substance which is a solvent of paraffin. The process is sometimes called " clearing," since many of the substances used for infiltration are also " clearing " agents.

The process of saturation should be carefully performed with well-dehydrated objects in the manner described in § 155.

Saturation liquids being liquids that are, on the one hand, miscible with alcohol, and on the other hand good solvents of paraffin, are not quite as numerous as could be wished.

According to Graefe (*Chem. Centralb.*, 1906, p. 874), at 20° C. petroleum ether (1 c.c.) dissolves 200 mg. of paraffin, chloroform 246, benzol 285, carbon tetrachloride 317. And according to Apáthy, at 20° C. benzol dissolves 8 parts per cent., chloroform 10, toluol 10, xylol 12, oil of turpentine 8, cedar oil 4 to 6, bergamot oil 0·5 to 3, creosote and clove oil hardly any. Acetone, according to Mayer, dissolves hardly any.

As a general thesis the best of all these are benzol or carbon bisulphide chloroform (for small objects), methyl benzoate-benzol and cedar oil.

Turpentine we do not recommend, because in our experience it is of all others the clearing agent that is the most *hurtful to delicate structures.*

Clove oil mixes very imperfectly with paraffin, and quickly *renders tissues brittle.*

Oil of bergamot mixes still more imperfectly with paraffin.

Benzol has been recommended by Brass (*Zeit. wiss. Mik.*, ii, 1885, p. 301), and is now much used.

Toluol (or toluen) has been recommended by Holl (*Zool. Anz.*, 1885, p. 223).

Chloroform is *deficient in penetrating power*, so that it requires an excessive length of time for clearing objects of any size; and it must be very thoroughly got rid of by evaporation in the paraffin bath, or by successive baths of paraffin, as if the least trace of it remains in the paraffin used for cutting it will make it soft. The process of removal requires a very long time, in some cases days. It ought therefore to be reserved for small and easily penetrable objects.

Naphtha has been recommended by WEBSTER (*Journ. Anat. and Physiol.*, xxv, 1891, p. 278).

FIELD and MARTIN (*Zeit. wiss. Mik.*, xi, 1894, p. 10) recommend a light petroleum known as "petroleum-æther." It is highly volatile, and thus a cause of shrinkage.

Sulphide of carbon has been recommended by HEIDENHAIN (*Zeit. wiss. Mik.*, xviii, 1901, p. 166) as being a very powerful solvent of paraffin. Most workers have found it to be much too disagreeable and dangerous a reagent for ordinary work, and *not necessary* even for delicate work. See under "Teeth" and "Chitin."

Carbon tetrachloride has been recommended by PLEČNIK (*op. cit.*, xix, 1903, p. 328) and PRANTER (*ibid.*, p. 329) on the ground of not dissolving out osmium-blackened fats.

MAYER finds it no better than benzol.

Cedarwood oil is, according to continued experience, for the reasons stated by LEE in *Zool. Anz.*, 1885, p. 563, for general work the *very best* clearing agent for paraffin imbedding. It penetrates rapidly, preserves delicate structure better than any clearing agent known, does not make tissues brittle, even though they may be kept for weeks or months in it, and has the great advantage that if it be not entirely removed from the tissues in the paraffin bath it will not seriously impair the cutting consistency of the mass; indeed, it sometimes improves it by rendering it less brittle. As has been mentioned above (§ 131), methyl benzoate-benzol is now being used a good deal instead of the cedar oil method. It should be noted that care must be taken to buy a high quality of cedar oil.

Xylol certainly causes shrinkage when used alone. It is excellent practice to clear first in cedarwood oil and then wash out in benzol or xylol, or to use the methyl benzoate-benzol method (§ 131).

159. The Paraffin Bath. The objects having been duly saturated with a solvent, the next step is to substitute melted paraffin for the saturating medium.

Some authors lay great stress on the necessity of making the passage from the saturating agent to the paraffin as gradual as possible, by means of successive baths of mixtures of solvent and paraffin kept melted at a low temperature, say 35° C. With *oil of cedar*, at all events, this is not necessary. We simply put the objects into melted paraffin kept just at its melting-point, and keep them there till they are thoroughly saturated; the paraffin being changed once or twice for fresh only if the objects are sufficiently voluminous to have brought over with them a notable quantity of clearing agent. If the objects have been for a very long time—months or years—in the cedar oil so that this has become thick Lee removes it partially or entirely by soaking in xylol (thirty minutes to several hours) before putting into the paraffin. But with fresh oil of cedar he finds no advantage in doing so. (See, however, § 808.)

GIESBRECHT's method (*Zool. Anz.*, 1881, p. 484), is as follows :—Objects to be imbedded are saturated with *chloroform*, and the chloroform and objects are *gradually warmed* up to the melting-point of the paraffin employed, and during the warming small pieces of paraffin are by degrees *added to the chloroform*. So soon as it is seen that no more bubbles are given off from the objects, the addition of paraffin may cease, for that is a sign that the paraffin has entirely displaced the chloroform in the objects. This displacement having been a *gradual* one, the risk of shrinkage of the tissues is reduced to a minimum.

MAYER (*Grundzüge*, LEE and MAYER, 1910, p. 84) first saturates the objects with *benzol*, and then adds to the benzol some small pieces of paraffin, and lets them dissolve in the cold. After several hours (up to eighteen) the whole is brought in an open vessel on to the cold water-bath, the bath is then warmed gradually so as to attain a temperature of 60° C. in about two hours, and as fast as the benzol evaporates *melted paraffin is added* to it. Lastly, the paraffin is changed once before the definite imbedding. He rarely leaves objects overnight in the water-bath.

APÁTHY (*Mikrotechnik*, pp. 149, 150) first *clears with oil of cedar*, then brings the objects (by the process described, § 134) into a *solution of paraffin in chloroform* saturated at the temperature of the laboratory. The objects remain in the chloroform-paraffin solution for from one to three hours, without warming, until all the cedar oil is soaked out of them. The whole is then warmed on the water-bath or oven to a few degrees above the melting-point of the paraffin intended to be used for imbedding, and the object is brought into a mixture of equal parts of paraffin and chloroform, being suspended therein *near the top* on a bridge made of hardened filter paper (or in a special apparatus to the same end, not yet described). It remains in this mixture, at the temperature of the oven, for one to three hours, and lastly is brought (still on the paper bridge or in the apparatus) into pure paraffin, where it remains for half an hour to two hours.

DENNE (*in litt.*, 1907) points out that the objects ought *at first* to be *at the bottom* of the mixture. For this mixture is not a true solution, and the lower section of the contents of the tube is comparatively free from paraffin while the upper part is nearly pure paraffin. He moves the holder up in the tube at intervals, and the infiltration proceeds gradually with the minimum risk of shrinkage. Lastly, he removes the objects, on the holder, to the *top* of a tube of pure paraffin.

The practice of giving successive baths first of soft and then of hard paraffin, which has been frequently advised, appears to us *entirely illusory*.

It is important to keep the paraffin *dry*—that is, protected from vapour of water during the bath.

It is still more important to keep it *as nearly as possible* at *melting-point*. If it be heated for some time to a point much over its normal melting-point, *the melting-point will rise*, and you will end by having a harder paraffin than you set out with. And as regards the preservation of tissues, of course, the less they are heated the better. Overheating, as well as prolonged heating, tends, amongst other things, to *make tissues brittle*.

The *duration of the bath* must, of course, vary according to the size and nature of the object. An embryo of 2 to 3 mm. in thickness ought to be thoroughly saturated after an hour's bath, or often less. Many workers habitually give much longer baths, we think often longer than necessary. But some objects, such as ova of Crustacea, may require three or four days (HEIDECKE, *Jena Zeit.*, xxxviii, 1904, p. 506; MAYER, *Grundzüge*, LEE and MAYER, 1910, p. 85; BRINKMANN, *Mitth. Zool. Stat. Neapel*, xvi, 1903, p. 367, three to five days for uterus of Selachians; MÜLLER, *Arch. mikr. Anat.*, lxix, 1906, p. 3, for lungs of mammals; POSO, *Esperienze microtechniche*, Napoli, 1910, p. 29, five to twelve days for uterus and placenta of *Homo*). LEE takes as a guide, generally, the length of time the object has taken to clear in the cedar oil, assuming that the *warm* melted paraffin ought to penetrate at least as quickly as the *cold* oil; and then allowing somewhat longer, say as much again, in order to be on the right side.

160. Water-baths and Ovens. In practically all laboratories rather expensive metal imbedding baths are used. These are thermostatically controlled in various ways. A. CRAIG-BENNETT (*J. R. M. S.*, 1930) describes an admirable imbedding apparatus, consisting essentially of a wooden box containing a 100 watt electric lamp (with a dome shade). Beneath the shade is a tray of wide tubes of wax, so placed that only the top layer of the wax melts. The possibility of overheating the objects is eliminated. Craig-Bennett states that the wax melts quickly and the bath need only be turned on a short interval before use. The box has a lift-up front, and takes up little space. It should be excellent for research workers, and could be made by laboratory attendants.

It is important that the paraffin should not be exposed to a moist atmosphere whilst it is in the liquid state. If a water-bath be used for keeping it at the required temperature provision should be made for protecting the paraffin from the steam of the heated water.

A very convenient apparatus for this purpose is that of Paul Mayer, or "Naples water-bath," which will be found described at p. 146 of *Journ. Roy. Mic. Soc.*, 1883, or CARPENTER'S *The Microscope*, p. 452. An extremely simple stove, which any one can make for himself, is described in *Centralbl. Bakt.*, xlv, 1907, p. 191 (see *Journ. Roy. Mic. Soc.*, 1908, p. 109). For others, see the price lists of the instrument makers, especially JUNG, and GRÜBLER and HOLLBORN; and the descriptions in the technical journals.

161. Imbedding in Vacuo. There are objects which, on account of their consistency or their size, cannot be penetrated by paraffin in the ordinary way, even after hours or days in the bath. For such objects the method of imbedding *under a vacuum* (strictly, under diminished atmospheric pressure) renders the greatest service. It not only ensures complete penetration in a very short time—a few minutes—but it has the further advantage of *preventing any falling in of the tissues*, such as may easily happen with objects possessing internal cavities if it be attempted to imbed them in the ordinary way. It is realised by means of any arrangement that will allow of keeping paraffin melted under a vacuum.

That of Hoffmann is described and figured at p. 230 of *Zool. Anz.*, 1884. In this arrangement the vacuum is produced by means of a pneumatic water aspiration pump, the vessel containing the paraffin being placed in a desiccator heated by a water-bath and furnished with a tube that brings it into communication with the suction apparatus.

Francotte (*Bull. Soc. Belg. Mic.*, 1884, p. 45) produces the requisite vacuum by the condensation of steam.

Fol (*Lehrb.*, p. 121) employs the vacuum apparatus of Hoffmann, but simplifies the arrangement for containing the paraffin. The paraffin is contained in a stout test-tube furnished with a rubber stopper traversed by a tube that puts it into communication with the pump. The lower end of the test-tube dips into a water-bath. You pump out the air once or twice, wait a few minutes, then turn out the object with the paraffin (which by this time will have become abnormally hard), and re-imbed in fresh paraffin.

See also Pringle, in *Journ. Path. and Bacteriol.*, 1892, p. 117, or *Journ. Roy. Mic. Soc.*, 1892, p. 893 ; Kolster, in *Zeit. wiss. Mik.*, xviii, 1901, p. 170 ; Berg, *Zeit. wiss. Mik.*, xxvi, 1909, p. 209 ; Fuhrmann *ibid.*, xxi, 1904, p. 462 ; Kolmer and Wolff, *ibid.*, xix, 1902, p. 148 ; Gemmill, *Journ. Roy. Mic. Soc.*, 1911, p. 26.

162. Imbedding and Orientation. *As soon as* the objects are thoroughly saturated with paraffin they should be *imbedded* by one of the methods given above (§§ 155 *et seq.*) and the paraffin cooled as described next §.

But it may be desirable to have the object fixed in the cooled paraffin in a precisely arranged position, and, above all, in a precisely *marked* position. Very small objects may be oriented as follows :—The object is removed from the melted paraffin, and placed on a cylinder of solid paraffin. A needle or piece of stout iron wire is now heated in the flame of a lamp, and with it a hole is melted in the end of the cylinder ; the specimen is pushed into the melted paraffin, and placed in any desired position. The advantages of the method lie in the quickness and certainty with which it can be performed. In using the needle it is important *to melt as little paraffin as possible at one time*, in order that that which is melted may cool again as rapidly as possible.

Kerr (*Quart. Journ. Micr. Sc.*, xlv, 1901, p. 4) employs an electrically heated needle.

For the exact orientation of fairly large objects, such as embryos, it is helpful, in a darkened room, to apply a powerful beam of light which passes without much obstruction through the paraffin

and reveals faithfully the outlines of the specimen. A small arc lamp is recommended.

The method of PATTEN (*Zeit. wiss. Mik.*, xi, 1894, p. 13) is useful when one desires to orient large numbers of small objects. You get some writing paper of the sort that is made with two sets of raised parallel lines running at right angles to each other ("linen cloth paper"). Small strips are cut from this, and at suitable intervals along them small drops of a mixture of collodion and clove oil, of about the consistency of thick honey, are arranged close together along one of the ribs that run lengthwise. The objects to be imbedded are cleared in clove oil or oil of bergamot. They are taken one by one on the point of a knife, and after the excess of oil has been drawn off, are transferred each to a drop of the collodion mixture, in which they will stay in any required position. When half a dozen or more objects have been oriented in reference to the cross lines (which are to be parallel to the section planes) the whole thing is placed in turpentine. This washes out the clove oil and fixes the objects very firmly to the paper. The paper with the attached objects is now passed through the bath of paraffin and imbedded in the usual way. After cooling on water the block is trimmed and the paper peeled off, leaving the objects in the paraffin close to the under-surface of the block. This surface is now seen to be marked by the orienting lines of the ribbed paper, and also by any record numbers which may before imbedding have been written with a soft pencil on the paper.

KNOWER (*Journ. Morph.*, xvi, 1900, p. 507) takes *smooth* paper and engraves parallel lines on it with a needle, and takes xylol instead of turpentine.

A somewhat more complicated form of this process has been described by WOODWORTH, *Bull. Mus. Comp. Zool.*, xxxviii, vol. xxv, 1893, p. 45.

A similar process has also been described by FIELD and MARTIN in *Zeit. wiss. Mik.*, xi, 1894, p. 11, small strips of gelatin being used instead of paper.

MAYER also (*Grundzüge*, LEE and MAYER, 1910, p. 89) takes strips of photographic gelatin, and lets the collodion set in benzol.

HOFFMANN (*Zeit. wiss. Mik.*, xv, 1899, p. 312, and xvii, 1901, p. 443) takes, instead of the ribbed paper, glass slips ruled with a diamond, and completely imbeds the objects in large drops of clove oil collodion (equal parts), allowed to stand for twenty-four hours in an open vessel. The drops are caused to set in xylol. See also SAMTER, *ibid.*, xiii, 1897, p. 441 ; JORDAN, *ibid.*, xvi, 1899, p. 33 ; and PETER, *Verh. Anat. Ges.*, xiii Vers., 1899, p. 134.

ENTZ (*Arch. Protistenk.*, xv, 1909, p. 98) orients in clove oil collodion on a *cover-glass* coated with paraffin, and puts the whole into chloroform in which the mixture sets into a sheet which can be detached.

DENNE (*Journ. Appl. Mic.*, iii, 1902, p. 888) imbeds on disks of paper held at the bottom of glass tubes containing the paraffin by bent wires, by means of which a cylinder of paraffin containing the object may be lifted out as soon as cool.

WILSON (*Zeit. wiss. Mik.*, xvii, 1900, p. 169) makes orientation lines by imbedding alongside the objects strands of osmium-blackened nerve-fibres. See also a further development by Wilson, *ibid.*, xxvii, 1910, pp. 228 and 231.

163. Cooling the Mass. Whatever method of imbedding and orientation in the molten paraffin has been employed, the important point now to be attended to is that *the paraffin be*

cooled rapidly. The object of this is to prevent crystallisation of the paraffin (which may happen if it be allowed to cool slowly) and to get as homogeneous a mass as possible.

If the definite imbedding has been done in a watch-glass, hold it on the top of cold water until all the paraffin has solidified, and then let it sink to the bottom. When thoroughly cool, cut out blocks containing the objects. If the watch-glass has been smeared with a drop of a mixture of equal parts of glycerin and water before putting the paraffin into it, the solidified paraffin will generally detach itself in a single cake and float up in a few minutes, or hours at any rate. Do *not* attempt to remove it entire by warming the bottom of the watch-glass. Similarly with the paper trays or metal imbedding boxes. Or you may put them to cool on a cold slab of metal or stone.

SELENKA cools the mass by passing a stream of cool water through the imbedding tube described above (§ 156). MAYER cools the mass in the paraffin-tight moulds (§ 156) by passing cold water through a special movable water-bath, which allows of the arrangement of the objects by transmitted light under a dissecting microscope, see *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 ; *Intern. Monatsschr. Anat. Hist.*, iv, 1887, p. 39. A complicated apparatus for the same purpose is described by MEISSNER (*Zeit. wiss. Mik.*, xviii, 1902, p. 286). Similarly, HAHN, *ibid.*, xxv, 1908, p. 184, and KAPPERS, *ibid.*, xxiv, 1907, p. 254. See also FARKAS, *ibid.*, xxx, 1913, p. 168, for experiments on cooling methods.

The paraffin blocks with the objects are now mounted on the carrier of the microtome in position for cutting, and pared to the proper shape (next §). If any bubbles or cavities or opaque spots be present, prick with a heated needle till all is smooth and homogeneous. The same should be done if any cavities present themselves in the course of cutting. In bad cases, re-imbed.

164. Shape and Orientation of the Block of Mass to be cut. These differ accordingly as the cutting is done with a slanting knife or a square-set knife (see next §). In the first case, the block is best trimmed to a three-sided prism, and orientated as in Fig. 4, so that the knife enters it at the angle *a* and leaves it at the angle *c*. When the section is cut it will adhere to the knife only by the angle *c*, and can thus most readily be removed by means of a brush or needle. The object itself should come to lie in the block close to the line *b c*, so that the knife at first cuts only paraffin, and that if the section begins to *roll* it may be caught and held down by a brush or section-stretcher before the object itself is reached. For the square-set knife the block is best trimmed to a four-sided prism, and orientated as in the first case, so that the knife first touches one angle, if only *isolated* sections are to be cut. But if *ribbons* (§ 174) are to be cut, the block must be orientated with one of its sides parallel to the knife-edge, and the opposite side must be strictly parallel to this one.

An object which is not approximately isodiametrical but gives a section which is wider in one direction than another should be orientated *end on*, that is, so as to present its narrowest diameter to the knife-edge; for it is in this position that it will offer the least resistance to the blade, and tend the least to make the edge bend away or dig into it. This is specially important with *longitudinal* sections of worms, *Amphioxus*, embryos of vertebrates, and the like. Most especially with a square-set knife should the narrowest diameter of the object be presented to the knife; and only when the object is particularly hard, or otherwise difficult to cut, should it be turned so as not to let the whole of that diameter be attacked at once by the knife, but only a corner of it. And as far as possible arrange that the hardest part of an object be the last to be touched by the knife.

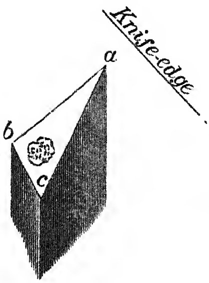


FIG. 4.

For NOACK's simple apparatus for accurately orientating small blocks, see *Zeit. wiss. Mik.*, xv, 1899, p. 438, or *Journ. Roy. Mic. Soc.*, 132, 1899, p. 550.

For ETERNOD's machine for trimming blocks to true cubes, see *Zeit. wiss. Mik.*, xv, p. 421, and for that of SCHAFFER, *ibid.*, xvi, 1900, p. 417.

165. Knife Position. The position to be given to the knife may be considered under two heads, viz., its *slant* and its *tilt*.

By the *slant* of the knife is meant the angle that its edge makes with the line of section: that is, with the line along which it is drawn through the object (or along which the object moves across it in the case of microtomes with fixed knives). The position is *transverse* when the edge makes an angle of 90° with the line of section, or the knife in that case is said to be set square. It is *oblique* or *slanting* when it makes a smaller angle with that line. The difference between the *effect* of the two positions is that the oblique position affords a *more acute-angled wedge* than the transverse one.

It does so for the following reasons: Neglecting for the moment the distinction between the cutting-facets and the surfaces of the blade (which are distinct usually because they are not ground to the same angle),* it is clear that the knife itself is a wedge, the

* The edge of a microtome knife is composed of two plane surfaces—the upper and lower cutting-facets, which meet one another at an acute angle, the cutting-edge, and posteriorly join on to the upper and lower surfaces of the blade (see some good figures of differently shaped knives in BEHRENS, KOSSEL und SCHIEFFERDECKER, *Das Mikroskop.*, pp. 115, *et seq.*; and in APÁTHY's paper quoted below). It will be seen that the two facets together form a wedge welded on to the blade by the base.

angle of which depends on the relation between the height of its base and the distance from the base to the edge. With the same base the angle becomes more acute the greater the distance from edge to base. Now by slanting the knife we can effect what is equivalent to an increase in the distance from edge to base; for we can thus increase the distance between the point of the edge which first touches the object, and the point of the back (strictly, of the back edge of the under cutting-facet) which last leaves it. When the knife is set transversely, the line along which any point of it traverses the object is the shortest possible from edge to base of the wedge, and the *effective* angle of wedge is the least acute obtainable with that knife. But if it is set obliquely as possible, the line along which any point of it traverses the object, traverses the knife from heel to toe, that is, along the greatest possible distance from edge to base, and therefore affords *practically* a much more acute angled wedge than in the first case; and so on, of course, for intermediate positions. (See the sterometrical constructions of these relations by SCHIEFFERDECKER, *op. cit.*, p. 115; and also with more instructive figures, APÁTHY, "Ueber die Bedeutung des Messerhalters in der Mikrotomie," in *Sitzber. med.-naturw. Section d. Siebenbürgischen Museumvereins*, Bd. xix, Heft 7, p. 1 (Kolozsevár, 1897, A. K. Ajtai).

For *honing* knives see SSOBOLEW, *Zeit. wiss. Mik.*, xxvi, 1909, p. 65; LENDVAI, *ibid.*, p. 203; FUNCK, *ibid.*, xxvii, 1910, p. 75.

166. Very large objects are best cut with the slanting knife, and so are all objects of very heterogeneous consistency, such as tissues that contain much chitin or much muscular tissue; and better with a slowly working sliding microtome than with a quick-working Rocker or the like. Soft masses such as gelatin or celloidin cut wet, can *only* be cut with the slanting knife. The slanting position causes less *compression* of sections than the transverse one. It has the defect of producing rolling in paraffin sections more easily than the transverse position. The latter is the proper position for cutting *ribbons* of sections from paraffin.

167. By the tilt of the knife is meant the angle that a plane passing through its back and edge makes with the plane of section: or, practically, the greater or less degree of elevation of the edge above the back (it is not to be confounded with the inclination of the long axis of the knife to the horizon; any accidental inclination that this may have is a matter of no moment).

The question of the proper tilt to be given to the knife under different circumstances has been investigated by APÁTHY, *loc. cit. supra*. He concludes—(1) The knife should always be tilted somewhat more than enough to bring the back of the under-cutting facet clear of the object. (2) It should in general be less

tilted for hard and brittle objects than for soft ones; therefore, *cæteris paribus*, less for paraffin than for celloidin. (3) The extent of useful tilt varies between 0° and 16° or occasionally 20°. (4) Excessive tilt causes rifts (longitudinal) in the paraffin, also furrows that in bad cases split up the section into narrow ribbons. It also makes sections roll. Also it may cause the knife not to bite, thus causing sections to be missed. Or it may give an undulatory surface to the sections, owing to vibrations set up in the knife, which may be heard as a deep humming tone. Further, we would add, excessive tilt may cause the knife to act as a scraper, carrying away portions of tissue bodily from their places. Excessive tilt may often be recognised by the knife giving out a short metallic sound just as it leaves the object. For knives with plane under-surfaces it is seldom advisable to give less than 10° tilt. Knives with concave under-surfaces, on the contrary, may require to be placed almost horizontal. Jung's knife-holders give mostly a tilt of about 9°, which is only enough for cutting ribbons with hard paraffin.

A knife with too little tilt will often cut a second section, or fragments of one, without the object being raised, showing that during the first cut the object was pressed down by the knife and recovered itself afterwards. This fault is denoted by the ringing tone given out by the knife on passing *back* over the object before the latter is raised. Such a knife gives out a dull rattling sound whilst cutting. Too little tilt causes folding or puckering of sections, and does not allow of the cutting of the thinnest possible sections, as the edge does not bite enough. It is thus frequently a cause of sections being missed, or coming off thicker at one end than the other.

A slanting knife should have more tilt given to it than a square-set one.

Ribbon section-cutting (§ 174) requires a relatively hard paraffin and less tilt. With celloidin it is very important to avoid insufficient tilt, as the elastic celloidin yields before an insufficiently tilted knife and is not cut.

The tilt of the knife is given to a certain extent by the knife-holder sold with the microtome. With plane-concave knives it can be regulated to a certain extent by simply turning the blade over. It is more accurately *regulated* by means of mechanical contrivances, of which the most simple are the horseshoe-shaped wedges of NEUMAYER (see Jung's price list). A pair of these, each ground to the same angle, is taken, and one of them placed (thin end towards the operator) under, and the other (thick end towards the operator) over, the clamping-arm of the knife-holder. Three pairs, having different degrees of pitch, are supplied, and are sufficient for most work. Other contrivances to the same end consist of knife-holders that permit of rotating the knife on its

long axis, and though more costly, will be found a great convenience where much section-cutting has to be done.

168. Safety Razor Blade Holders. We have used one of these supplied by Leitz. It performed quite well, and is a solution of the difficulty of providing microtome knives for classes of students.

169. Cutting and Section-stretching. Paraffin sections are cut *dry,*—that is, with a knife not moistened with alcohol or other liquid. By this means better sections are obtained, but a difficulty generally arises owing to the tendency of sections so cut to curl up on the blade of the knife. It is sometimes difficult by any means to unroll a thin section that has curled. To prevent sections from *rolling*, the following points should be attended to.

First and foremost, the paraffin *must not be too hard,* see § 159.

If, after cutting has begun, the paraffin be found to be too hard, it may be softened by placing a lamp near the imbedded object. But then, the paraffin being warmed most on the side nearest the lamp, becomes softer on that side, and the sections have a tendency to become compressed and puckered-in on that side.

If, on the contrary, the paraffin be found too soft, it may be hardened by exposing it to the cooling influence of a lump of ice.

It is often sufficient to moderate the temperature of the room by opening or closing the window, stirring the fire, setting up a screen, or the like.

For other devices for warming or cooling the paraffin see HELD, *Arch. Anat. Phys., Anat. Abth.*, 1897, p. 345 ; VAN WALSEM, *Zeit. wiss. Mik.*, xi, 1894, p. 218 ; LENDENFELD, *ibid.*, xviii, 1901, p. 18 ; KRAUSE, *ibid.*, xxv, 1908, p. 299 ; FOOT and STROBELL, *Biol. Bull. Wood's Hole*, ix, 1905, p. 281.

Secondly, the knife should be set square, for the oblique position encourages rolling, and the more the knife is oblique the more do the sections roll.

Thirdly, it is better to cut ribbons than disconnected sections ; ribbons of sections will often cut flat, when the same mass will only give rolled sections if cut disconnectedly.

Rolling may often be lessened or suppressed by cutting the sections thinner.

Mechanical means may be employed. The simplest of these is as follows :

During the cutting the edge of the section that begins to curl is caught and held down on the blade of the knife by means of a small camel-hair brush with a flat point, or by a small spatula made by fixing a piece of paper on to the back of a scalpel. Or, which is much better, the section is held down by means of an instrument called a " section-stretcher." This consists essentially of a little metallic roller suspended over the object to be cut in such a way as to rest on its free surface with a pressure that can be delicately regulated so as to be sufficient to keep the section flat without in any way hindering the knife from gliding beneath it.

170. See the descriptions of various forms of section-stretchers, *Zool. Anzeig.*, vol. vi, 1883, p. 100 (SCHULTZE); *Mitth. Zool. Stat. Neapel*, iv, 1883, p. 429 (MAYER, ANDRES, and GIESBRECHT); *Arch. mik. Anat.*, xxiii, 1884, p. 537 (DECKER); *Bull. Soc. Belg. Mic.*, x, 1883, p. 55 (FRANCOTTE); *The Microscope*, February, 1884 (GAGE and SMITH); WHITMAN'S *Meth. in Mic. Anat.*, 1885, p. 91; *Zeit. wiss. Mik.*, iv, 1887, p. 218 (STRASSER); *ibid.*, x, 1893, p. 157 (BORN). The best are those of Mayer and Born.

Another plan is to allow the sections to roll, but to control the rolling. To this end, the block of paraffin is pared to the shape of a wedge five or six times as long as broad, the object being contained in the broad part, and the edge turned towards the knife (see Fig. 4). The sections are allowed to roll and come off as coils, the section of the object lying in the outermost coil, which will be found to be a very open one—indeed, very nearly flat. Lay the coil on a slide with this end downwards, warm gently, and the part containing the object will unroll completely and lie quite flat.

ANILE (*Glandole duodenali*, Napoli, 1903, p. 51) and VASTAINI-CRESI (*Mon. Zool. Ital.*, 1906, p. 164) lay a strip of wet filter-paper on the block.

Another defect is the *compression* and the crumpling or puckering of sections, indicating that the paraffin has been compressed by the knife instead of being merely cut true by it. Such sections, besides showing creases or folds, have a smaller area than that of the block from which they are cut. This is a bad fault, for the compression may obliterate important cavities or efface important limits between cell-layers, etc. It may be caused by a badly cutting knife, and is very easily caused by the paraffin being too soft. To prevent it, correct the knife or cool the paraffin, or re-imbed in harder paraffin.

Very large sections tend to form folds *on the knife*, and are difficult to remove from it. MAYER (*Grundzüge*, LEE and MAYER, p. 94) gets them to wrap themselves round a glass or gelatin tube laid on the block just in front of the knife-edge and rolled forwards as it progresses. When cut, the section is rolled off on to the surface of water.

171. Cutting Brittle Objects. Some objects are by nature so brittle that they break or crumble before the knife, or furnish sections so friable that it is impossible to mount them in the ordinary way. Ova are frequently like this. One remedy consists in covering the exposed surface of the object just before cutting each section with a thin layer of collodion, which serves to hold together the loose parts; and will enable the operator to *cut sections considerably thinner* than can be obtained in the usual way.

MARK (*Amer. Natural.*, 1885, p. 628; cf. *Journ. Roy. Mic. Soc.*, 1885, p. 738) gives the following directions:

"Have ready a little very fluid collodion in a small bottle, through the cork of which passes a small camel-hair brush, which just dips into the collodion with its tip. The collodion should be of such a consistency that when applied in a thin layer to a surface of paraffin it dries in two or three seconds without leaving a shiny surface. It must be diluted with ether as soon as it begins to show signs of doing so.

"Take the brush out of the collodion, wipe it against the neck of the bottle, so as to have it merely moist with collodion, and quickly pass it over the free surface of the preparation. Care must be taken not to let the collodion touch the vertical surfaces of the paraffin, especially not the one which is turned towards the operator, as that will probably cause the section to become stuck to the edge or under-surface of the knife. As soon as the collodion is dry, which ought to be in two or three seconds, cut the section, withdraw the knife, and pass the collodion brush over the newly exposed surface of the paraffin. Whilst this last layer of collodion is drying, take up the section from the knife and place it with the collodionised surface downwards on a slide prepared with fixative of Schaellibaum. Then cut the second section, and repeat the manipulations just described in the same order."

HENKING (*Zeit. wiss. Mik.*, iii, 1886, p. 478) takes instead of collodion a solution of *paraffin* in absolute alcohol.

For extremely brittle objects, such as ova of Phalangida, he recommends a thin (light yellow) solution of *shellac* in absolute alcohol.

HEIDER (*Embryonalentw. v. Hydrophilus*, 1889, p. 12; cf. *Zeit. wiss. Mik.*, viii, 1892, p. 509) employs a solution made by mixing a solution of *gum mastic* in ether, of a syrupy consistency, with an equal volume of collodion, and diluting the mixture with ether until quite thin and liquid.

RABL (*ibid.*, xi, 2, 1894, p. 170) employs *superheated paraffin* (of about 100° C.). This has the advantage of filling up any cavities there may be in the objects, and also of *preventing the sections from rolling*. A complicated development of this process is described by LENDENFELD in *Zeit. wiss. Mik.*, xviii, 1901, p. 18.

APÁTHY (*Mikrotechnik*, p. 183) employs a 1 per cent. solution of celloidin, allows the sections to roll, and unrolls them by the water-process (§ 175).

JORDAN (*Zeit. wiss. Mik.*) adds 5 drops of oil of cedar to 15 c.c. of the solution of celloidin, and finds that rolling is prevented.

172. Collodionisation, as it has been called, is merely a palliative. One may very aptly cite the old adage that "an ounce of prevention is worth a pound of cure." With the methods now available there is little excuse for attempting to cut hard or brittle material for if the proper technique is employed in the first place nearly all objects can be sectioned without the difficulties experienced by earlier workers.

Hardness and brittleness may be due to a number of causes. *First* is the selection of an improper fixation method. The reader will find in the various chapters of this book, adequate directions for the best type of fixing fluid to be used for different animal and plant tissues. If he lacks experience in these methods he should read the proper section before attempting to fix a new type of tissue. *Second,* ethyl alcohol has a tendency to harden tissues

and difficult material should not be left in the higher grades any longer than is necessary for dehydration. *Third*, some of the clearing agents render certain types of material both hard and brittle. *Fourth*, leaving tissues too long in paraffin or overheating them may cause brittleness. *Fifth*, certain types of tissues, even in the hands of the most experienced worker, are always hard and brittle when treated by ordinary methods. In these cases one should try one of the methods which are described below :—

173. A. Johnston's Rubber Paraffin. (*Jour. Appl. Micr.*, vi, 1903, p. 2662).

Crude india-rubber, cut in small pieces .	1 part by weight.
Paraffin, melted and tinged an amber colour with asphalt	99 parts.

Heat to 100° C. twenty-four to forty-eight hours. If a lower temperature is used treat the paraffin for a week or ten days. Then pour off the supernatant fluid and cool at once. Use ordinary paraffin, but note that if you allow rubber paraffin to stand melted in the oven for a long time it tends to decompose.

Johnston's rubber paraffin is, perhaps, the most valuable aid we have for sectioning tissues which would be hard and brittle in ordinary paraffin.

B. The N-Butyl Alcohol Method. This method is useful when one wishes to avoid the hardening effect of ethyl alcohol and such a clearing agent as xylol. (See § 127.)

N-butyl alcohol is only slightly soluble in water, but mixes readily with the higher grades of ethyl alcohol and also with paraffin, so that no separate clearing agent is needed.

The N-butyl alcohol method was originally used for making sections of woody tissues which were too hard for ordinary methods but is now recommended for making sections of lightly chitinised insects, and promises to be a very useful adjunct for difficult material. For details refer to § 127, p. 63.

C. Dioxan Method. This method also avoids the use of ethyl alcohol and benzol or xylol. Refer to § 130, p. 64.

D. Hardening Wax by Ceresin Method. This method enables one to harden the wax and to get something approaching the celloidin-wax method. It has been recently used a good deal. See § 177.

E. Double Imbedding in Paraffin Wax and Celloidin. See § 190.

F. Treatment with Phenol. Phenol has long been known to impart to tissues an elastic texture and recently several methods have been developed which employ this agent to soften tissues which would otherwise be hard and brittle on sectioning.

PETRUNKEVITCH (*Science*, lxxvii, 1933, p. 117) uses phenol directly with the fixative and recommends the following :—

Stock solution A :

Distilled water	100 c.c.
Nitric acid, c.p.	12 ,,
Cupric nitrate, c.p.	8 grm.

Stock solution B :

80 per cent. alcohol	100 c.c.
Phenol, crystals, c.p.	4 grm.
Ether	6 c.c.

Take 1 part of solution A and 3 parts of solution B. Fix immediately as this mixture does not keep well. Twelve to twenty-four hours is usually sufficient, but in no case should it exceed forty-eight hours. Wash in several changes of 70 per cent. alcohol.

SLIFER and KING (*Science*, lxviii, 1933, p. 366) have found that tissue fixed by some other method and subsequently treated with a 4 per cent. solution of phenol dissolved in 80 per cent. alcohol very effectively softens the yolk of a grasshopper's egg so that it may be sectioned with very little difficulty.

174. Ribbon Section-cutting. If a series of paraffin sections be cut in succession and not removed from the knife one by one as cut, but allowed to lie undisturbed on the blade, it not infrequently happens that they adhere to one another by the edges so as to form a chain or ribbon which may be taken up and transferred to a slide without breaking up, thus greatly lightening the labour of mounting a series. For the production of a ribbon, the paraffin must be of a *melting-point* having the right relation to the temperature of the laboratory, see § 169. Secondly, the *knife should be set square.* Thirdly, the block of paraffin should be trimmed so as to present a straight edge parallel to the knife-edge ; and the opposite edge should also be parallel to this. It is by no means necessary to have recourse to special mechanical contrivances, as in the so-called ribbon microtomes ; the Thoma microtome is sufficient. But the automatic microtomes, and amongst them the Cambridge Rocking Microtome and the Minot, are certainly most advantageous for this purpose

If the paraffin is very hard, it is *necessary* for sections of 10 μ, and advisable for thinner ones, to *coat the block* with softer paraffin. To do this, take paraffin of about 40° C. melting-point, melt it, heat it to about 80° on the water-bath, dip the block into it for an instant, and rapidly turn it over so that the fluid paraffin may run down away from the top part as much as possible. Allow it to cool, and pare away again the soft paraffin from the two sides that are not to be arranged parallel to the knife. Or,

as we frequently prefer, simply plaster a wall of soft paraffin (superheated) on to the upper and lower faces of the block with a small spatula. Large blocks may have two coatings given them.

It sometimes happens that the ribbon becomes *electrified* during the cutting, and twists and curls about in the air in a most fantastic and undesirable manner. It may be got flat by warming slightly. Professor Teacher tells us that it is a great help to breathe on the razor as the sections are coming off, the effect being to reduce electrification.

175. Section Flattening. The sections having been obtained may be cleared and mounted at once if they are quite perfect, that is, neither rolled nor creased nor *compressed.* But should they *in the least degree* show any of these defects, they must first be unrolled or smoothed, or *expanded* to their proper dimensions.

The most efficacious plan is combined treatment with fluid and heat. The sections are either floated on to the surface of warm water or warm alcohol contained in a suitable dish, which causes them to flatten out perfectly, and are then transferred to a slide, by floating them into position, or otherwise. Or the slide has a layer of water spread over it, the sections are laid on the water, and the slide is heated (to somewhat *below* the melting-point of the paraffin) until the sections flatten out, which happens in a few seconds.

A special water-bath for flattening sections is described by NOWAK in *Zeit. wiss. Mik.*, xii, 1896, p. 447.

176. Clearing and Mounting. The sections having been duly smoothed by one of these processes, and duly fixed to the slide (Chapter XI), unless it is desired to keep them loose, all that now remains is to get rid of the paraffin and mount or stain as the case may be. Many solvents have been recommended for this purpose :—Turpentine, warm turpentine, a mixture of 4 parts of essence of turpentine with 1 of creosote, creosote, a mixture of turpentine and oil of cloves, benzol, toluol, xylol, thin solution of Canada balsam in xylol (only applicable to very thin sections), hot absolute alcohol, naphtha, or any other paraffin oil of low boiling-point. Of these xylol and toluol are generally in most respects the best. Benzol and chloroform are too volatile for safe manipulation.

If the slide be warmed to the melting-point of the paraffin, a few seconds will suffice to remove the paraffin if the slide be plunged into a tube of xylol or toluol. For *thin* sections, 10 to 15 μ, it is *not necessary to warm* at all. The sections may be mounted direct from the xylol, or the slide may be brought into a tube of alcohol to remove the solvent for staining.

Paraffin sections *can* be stained without removal of the paraffin, so that after-treatment with alcohol can be suppressed, but this is only very exceptionally advantageous.

177. Pure Paraffin. It is now almost universally admitted that pure paraffin is superior for ordinary work to any of the many mixtures with wax and the like that used to be recommended. Paraffin varies enormously in hardness according to the temperature of its surroundings. It should therefore be taken of a melting-point suitable to the temperature of the laboratory. *A paraffin melting at* 50° C. or a little harder, is that which in our experience gives the best results *so long as the temperature of the laboratory is between* 15° and 17° C. For higher temperatures a harder paraffin is required, and for lower temperatures a softer one.

Many workers of undoubted competence prefer masses somewhat harder than this ; so, for instance, Heidenhain (58°), Apáthy (55°), Rabl (56°), Mayer (58° to 60° in summer ; in winter about 56°, but never less than 50°). Mayer points out that at Naples the temperature during five months of the summer and autumn is over 22° C. in the laboratory, sometimes over 30°. Temperatures such as these are seldom realised in the British Isles, and, whilst we quite admit that such hard paraffin may have its *raison d'être* for Naples, we hold that for that very reason it is in general unnecessarily hard for cooler climates.

Our recommendation of a relatively soft paraffin refers to work with the Thoma sliding microtome. Microtomes with *fixed* knives, such as the Cambridge, the Minot, or the Reinhold-Giltay, will give good results with much harder paraffin, and, in fact, require such.

Stout knives of hard steel will take a harder paraffin than thin ones of soft steel; but the latter may be preferable for soft masses.

For *thin sections* a harder paraffin is required than for thick ones.

Hard objects require a harder paraffin than soft ones.

Brass (*Zeit. wiss. Mik.*, ii, 1885, p. 300) recommends paraffin that has been kept for some years, as it has less tendency to crystallise than new paraffin.

Paraffin of various melting-points is easily obtainable. Intermediate sorts may be made by mixing hard and soft paraffin. Lee finds that 2 parts of paraffin melting at 50° with 1 of paraffin melting at 36° C. give a mass melting at 48° C., and a mixture of 1 part of that melting at 53° with 1 part of that melting at 45° gives a mass melting at 50° C.

According to E. Burchardt (*Jena Zeit. Naturw.*, xxxiv, 1900, p. 719) *mixtures* of paraffins of different melting-points give better results than an unmixed paraffin of the same melting-points as the mixture. He recommends 10 parts of 40° paraffin + 1 of 45° + 1 of 52° + 1 of 58° + 6 of 60°.

For methods for ascertaining melting-points see KISSLING, *Chem. Centralb.*, ii, 1901, p. 507.

Ceresin Method of Higgs, Waddington and Kriebel. This method has been worked out independently by WADDINGTON and KRIEBEL (*Nature*, October 26th, 1935) and 'ESPINASSE (communicated), the former for use in sectioning hard embryonic material, the latter for developing feathers. That the addition of ceresin alters the physical structure of paraffin wax practically to micro-crystallinity was first pointed out by P. G. HIGGS in a paper read before the Institution of Petroleum Technologists in December 11th, 1934, and reported in *Nature*, January 19th, 1935. 'Espinasse obtained some ceresin and found it remarkably good for his work on feathers, while Waddington and Kriebel have used it for sectioning yolky eggs and embryos, and reported very favourably on it. Waddington uses various mixtures of ceresin and wax even as low as 0·5 per cent., with great benefit. Ceresin itself, or mixtures of ceresin and wax, with a high proportion of the former must be melted in a beaker in a vessel placed in hot water, as the temperature necessary is too high to be got with a normal oven. 'Espinasse used the following mixture: paraffin wax (M.P., 52° C.), Woolworth candles (suggested by Dr. Henderson of Hull) and ceresin (M.P. *circa* 72° C.), 1 part each. This makes a very hard mixture and should be filtered. The candles supplied by Messrs. Woolworth, Mr. 'Espinasse informs us, have a low melting-point, but add greatly to the smoothness of the mixture.

Methyl Benzoate Celloidin Ceresin Method of 'Espinasse (communicated). The methyl benzoate method of Peterfi has been combined with the ceresin method for cutting very hard objects. It will give *thin* sections of small crustacea developing feathers, etc., and in one case gave excellent sections of the human pituitary and infundibular region. Dissolve 10 grm. of perfectly dry celloidin in 1 litre of methyl benzoate. Use this for clearing from ethyl alcohol. Objects may be left weeks without hurt. Transfer to pure benzol for a few minutes in the case of small objects, or some hours for larger specimens. Bring into wax, using the mixture mentioned in the previous paragraph. Then prepare a very hard mixture (higher proportion of ceresin) heated in a beaker; this is poured into a mould, and when beginning to cool, it receives the object.

178. Overheated Paraffin. SPEE (*Zeit. wiss. Mik.*, ii, 1885, p. 8) takes paraffin of about 50° C. melting-point and heats it in a porcelain capsule by means of a lamp until it has become brownish-yellow, and after cooling shows an unctuous or soapy surface on being cut. This mass may be obtained ready prepared from Grübler. The object of this preparation is to make the mass stickier, in view of cutting ribbons.

VAN WALSEM (*Verh. Akad. Wetensch. Amsterdam*, 1899, p. 132) still recommends the addition of 5 per cent. of yellow wax to paraffin of 52° to 57° melting-point (for large sections of central nervous system).

GELATIN MASSES

179. Gelatin Imbedding is a method that has the advantage of being applicable to tissues that have not been *in the least degree dehydrated.*

The *modus operandi* is, on the whole, the same as for other fusion masses, with the difference that the objects are prepared by saturation with *water* instead of alcohol or a clearing agent. After the cooling of the mass it may sometimes be cut at once, but it is generally necessary to harden it. This may be done by treatment for a few minutes with absolute alcohol (KAISER), or for a few days with 90 per cent. alcohol (KLEBS) or chromic acid (KLEBS), or formaldehyde (NICOLAS), or it may be frozen (SOLLAS).

The mass can be removed from the sections by means of warm water.

Gelatin imbedding has been used by NICOLAS (*Bibliogr. Anat.*, 3, 1896, p. 274), OLT (*loc. cit.*), and GASKELL (*J. Path. Bact.*, 1912, p. 58) to improve the freezing technique, but these workers had trouble in the subsequent handling of the tissues. ZWEMER (*Anat. Rec.*, 57, 1933, p. 41) has improved on the earlier methods and his technique is given below :—After fixing in formalin, or some other way, the tissue is washed and then placed in a 5 per cent. solution of gelatin in an incubator at about 37° C. for twenty-four hours. It is then placed in a 10 per cent. solution of gelatin at the same temperature for about twelve to sixteen hours. The tissue is now imbedded in a 10 per cent. solution of gelatin, in a dish of convenient size and placed in a refrigerator for a few hours until the gelatin hardens. Blocks of gelatin containing the tissue are cut out and dropped into a 10 per cent. solution of formol and left for several hours to make the gelatin insoluble in water. They can be left in this solution indefinitely. Before sectioning the blocks are rinsed in water and trimmed down close to the tissue. They are then frozen in dry CO_2 gas until the blocks are a uniform white, and then they are allowed to thaw until the knife will cut real slices. Section rapidly while conditions are favourable. Sections as thin as 5 micra have been obtained by this procedure.

The sections may be transferred, with a camel-hair brush, to a dish of distilled water, if they are to be mounted immediately, or they can be kept indefinitely in a 10 per cent. solution of formol. Sections are transferred to a clean glass slide and after the water is removed a drop or two of 1 per cent. gelatin is run under the section. Now the slide is dried for five minutes at about 35° to 37° C. and then placed in a 10 per cent. solution of formol to fix the gelatin. After rinsing, the section is ready for staining as desired. Afterwards, a mounting medium called " Glychrogel "

is added to the section and the coverslip is put in place. After a few hours, the slip will be firmly fixed to the slide. Directions for making glychrogel are given in § 474.

180. **Other Glycerin Gelatin**, KLEBS' (*Arch. mik. Anat.*, v, 1869, p. 165). A concentrated solution of isinglass mixed with half its volume of glycerin.

KAISER'S (*Bot. Centralb.*, i, 1880, p. 25). One part by weight of gelatin is left for about two hours in 6 parts by weight of water; 7 parts of glycerin are added, and for every 100 grm. of the mixture 1 grm. of concentrated carbolic acid. The whole is warmed for ten to fifteen minutes, stirring all the while, until the whole of the flakes produced by the carbolic acid have disappeared.

GERLACH'S (*Unters. a. d. Anat. Inst. Erlangen*, 1884; *Journ. Roy. Mic. Soc.*, 1885, p. 541). Take gelatin, 40 grm.; saturated solution of arsenious acid, 200 c.c.; glycerin, 120 c.c. Clarify with white of egg. The objects to be prepared for imbedding by a bath of one-third glycerin.

APÁTHY (*Mitth. Z. Stat. Neapel*, xii, 1897, p. 718, and *Zeit. wiss. Mikr.*, xxix, 1913, p. 472) soaks *small* objects first in glycerin and water (equal parts) and then for at least twenty-four hours at 40° C. in a solution of 1 part of gelatin in 3 of glycerin and 6 of water. They are then arranged in some of this in an imbedding box, and the whole is warmed (over calcium chloride) in a stove at 45° to 60° C. until the mass has evaporated down to one-half, losing 5 of its 6 volumes of water (as we understand—the description is not clear). Blocks are then cut out and hardened in *absolute* alcohol (suspended therein) for several days (one day per millimetre of thickness), cleared in terpinol (one day per millimetre), and cut with a knife wetted with the same. Said to give sections of 3 μ, without the least shrinkage.

BRUNOTTI'S **Cold Gelatin Mass** (*Journ. de Botan.*, vi, 1802, p. 194). Twenty grm. gelatin dissolved with heat in 200 c.c. distilled water, and 30 to 40 c.c. of glacial acetic acid with 1 grm. corrosive sublimate added after filtering. Objects are prepared by soaking in some of the mass diluted with 2 to 3 volumes of water, then imbedded in the undiluted mass. The mass is then hardened in spirit of bichromate of potash, picric acid, or the like. No heat at all is required in this process.

NICOLAS'S **Method** (*Bibliogr. Anat.*, Paris, 3 année, 1896, p. 274). Preparations are first soaked for one or two days in a 3 to 4 per cent. aqueous solution of gelatin kept at 25° C., then for the same time in a 10 per cent. solution, and then for two or three days more in a 20 to 25 per cent. solution containing 8 to 10 per cent. of glycerin and kept at 35° C. They are then imbedded in some of the same mass in paper trays, and as soon as the gelatin has set are thrown into a mixture of formol 1 part, water 7. After a few days therein the gelatin has become hard and insoluble, and may be cut or preserved for months in weak formol solution, or dilute alcohol or glycerin, or even in pure water. Sections must be very gradually passed through successive alcohols for dehydration, as they curl up very easily. They however, flatten out at once on being brought from absolute

alcohol into cresylol, and may then be mounted in balsam. To mount in glycerin is of course easy.

Burzynski (*Polu. Arch. Boil. Med. Wiss.*, i, 1901, p. 39) finds that alkaline formol hardens gelatin better than acid.

Gaskell (*Journ. Path. Bact.*, July, 1912, p. 58) soaks in *pure* gelatin, melted *s.a.*, for two to five hours at 37° C., and hardens the mass in *vapour* of formol, for three or more days. To cut, he freezes. He mounts in glycerin jelly, to avoid dehydration and shrinkage.

CHAPTER X

CELLOIDIN * AND OTHER IMBEDDING METHODS

181. Introduction. Celloidin, Parlodion and Photoxylin are the chief commercial preparations of nitrocellulose which have been used for imbedding since DUVAL (*Journ. de l'Anat.*, 1879, p. 185) introduced his collodion method. In comparison with paraffin wax imbedding, the celloidin techniques possess several important advantages. The shrinkage and distortion, so common in paraffin sections and particularly in the case of large blocks of material, is minimised. Celloidin tends so often to be used only for tough or brittle tissues and it should be stressed that the minute details in the morphology and staining reactions of various cell-types, such as occur in hæmopoietic tissues, are more completely preserved in celloidin sections. The student should examine celloidin sections of lymph glands, for instance, and note the preservation of the lymphocytes which undergo marked shrinkage during paraffin imbedding. Owing to the elastic nature of celloidin, loose membranes and fragile or brittle areas in tissues are more easily kept intact in the sections. For this reason celloidin imbedding is invaluable for nervous tissues which have been made brittle by prolonged mordanting. It is not necessary, of course, to remove the transparent celloidin before mounting the sections. A few special tissues like tendon, fibrocartilage, nails and claws, together with the invertebrate forms possessing chitinous or horny parts, are seldom sectioned successfully without the use of celloidin. Much of the characteristic hardness of these special tissues, following paraffin imbedding, is due to the action of hot wax and can be avoided with the celloidin technique. Material cut in celloidin usually has a greater affinity for dyes as compared with paraffin sections. Whereas the majority of the routine staining techniques in this textbook are generally used with paraffin sections, a few, *e.g.* Mallory's triple connective tissue stain, were originally devised for celloidin sections and undoubtedly give more specific reactions in celloidin. Celloidin imbedding is essentially a technique for the critical histologist and embryologist who is concerned with these advantages and not with the main disadvantage of the time taken in preparing the sections.

182. Résumé of Celloidin Imbedding Techniques. The process of celloidin imbedding is open to more variations than paraffin wax

* K. C. R.

infiltration. Generally speaking, four distinct stages are involved after the fixation of the tissue.

1. Complete dehydration, followed by soaking the tissue in the celloidin solvent.

2. Infiltration of the tissue with increasing concentrations of celloidin, starting with a 2 per cent. solution.

(*a*) Infiltration at room temperature, or

(*b*) Infiltration under pressure at 50° to 60° C. (The hot celloidin technique).

3. Imbedding the tissue in concentrated celloidin (8 to 15 per cent.) followed by carefully controlled evaporation of the solvent so that the celloidin sets in a firm block, or

Imbedding the tissue in celloidin which is concentrated by adding fresh dry celloidin and heating under pressure until the imbedding mass becomes viscous.

4. Hardening of the block of precipitated celloidin to give it adequate rigidity for cutting the sections.

183. Preparation before Infiltration. Before proceeding with final dehydration in absolute alcohol, it is important to remove gases from such tissues as lung and from organs with cavities or extensive layers of loose connective tissue. The tissue in most cases can be placed in 70 or 90 per cent. alcohol under an exhaust pump but, where the rapid evolution of gas bubbles is likely to damage the tissue, the alternative method of using alcohols from which dissolved gases have been removed by heating is advised. If the piece of tissue contains large masses of adipose tissue it is better to extract the fat by transferring it to benzol and then returning it to several baths of absolute alcohol before proceeding with the celloidin infiltration.

The dehydration of the tissue must be very thorough. A few crystals of phenol added to the last two baths of absolute alcohol or the more elaborate methods of re-distilling the alcohol, using iodine and magnesium (Bjerrum and Lund, *Brit. Chem. Abstr.*, 1931, 461 A), may be useful. Celloidin infiltrates tissues successfully after most routine fixatives, with the special exception of Bouin's picro-formol-acetic mixture. Bolcek (*Zeit. wiss. Mikr.*, 47, 1930, p. 334) recommends placing Bouin fixed material for ten to forty-eight hours from 96 per cent. alcohol into :—

Cedarwood oil . .	10 c.c.
Absolute ethyl alcohol .	80 c.c.
Origanum oil . . .	20 c.c.
Nitric acid . . .	10 c.c.

The tissue is then washed for twenty-four to forty-eight hours in 96 per cent. alcohol and is ready for complete dehydration and infiltration.

After dehydration the tissue is placed in equal parts of absolute ethyl alcohol and ethyl ether (0·72, water free), the celloidin

solvent. Very small pieces of tissue will require only one hour's soaking or they may be transferred direct from absolute alcohol to the celloidin solution.

184. Infiltration. Celloidin may be obtained in solutions of various concentrations (Gurr) or in the form of shreds stored in water (Schering). The pure celloidin shreds must be washed in absolute alcohol and dried at room temperature on thick filter paper. It is convenient to make up two solutions, 8 and 16 per cent., from which the intermediate concentrations can be quickly made by dilution with the solvent. The dry celloidin is weighed and placed overnight in a volume of absolute alcohol equal to half the final volume of solvent. The celloidin swells, and when the remaining volume of ether is added the celloidin dissolves after a further twenty-four hours. Celloidin solutions should be stored in double stoppered, wide mouthed bottles to prevent the escape of ether (B.P. 34° C.) and to facilitate the pouring of thick solutions. Storage in a refrigerator will minimise the evaporation of the solvent in hot weather.

185. Infiltration at Room Temperature. The piece of tissue is placed successively in 2, 4, 6 and 8 per cent. celloidin for periods extending from several days to one month in each solution. It is impossible to state the time required for infiltration in any but these vague periods as so much depends on the volume of the material and the density of the various tissues involved. The 6 per cent. solution may be omitted for small pieces of tissue and, generally speaking, the time can be cut down with more advantage in the 8 per cent. than in the 2 and 4 per cent. solutions. Heavily mordanted material requires prolonged infiltration (*e.g.* nervous tissue, Weigert-Pal technique).

186. Infiltration at 50°–60° C. Hot celloidin solutions are not apparently injurious to delicate cytoplasmic structures and do not cause increased shrinkage or hardening.

The tissue is placed in 2 per cent. celloidin in a stout glass bottle fitted with a heavy cork which is wired securely to the neck of the bottle, see Walls (*Stain Tech.*, vii, 1932, p. 135.). The bottle is incubated for twelve to twenty-four hours at 50° to 60° C. and the pressure of the ether and alcohol vapour forces the celloidin into the tissue. A minimum of celloidin solution should be used to give sufficient space in the bottle in which to develop maximum pressure. The bottle can be inverted to prevent the escape of ether through the cork. When the bottle is removed from the incubator it is allowed to cool to room temperature before removing the cork. The 2 per cent. celloidin is then replaced with 4 per cent. and the cork rewired on to the bottle. After a further twelve to twenty-four hours incubation, the process is repeated for the 6 and 8 per cent. solutions. The method is quite a safe one if reasonable care is taken, but it is essential

not to loosen the cork while the celloidin is above room temperature, otherwise the tissue will be ruined by the rapid formation of ether-alcohol bubbles released when the pressure is suddenly reduced.

The rapid infiltration of this newer method which has been adapted from the more elaborate botanical techniques, see WETMORE (*Stain Tech.*, vii, 1932, p. 37), is a great advantage, but it is possible, in a few special cases, such as the Organ of Corti in adult mammals, that heating may cause distortion of delicate structures which is not found after infiltration at room temperature.

187. Imbedding. The aim in imbedding is to cast the tissue in a solid block of celloidin which is concentrated to a degree so that it no longer responds to squeezing or retains a finger-print impression. It must be emphasised immediately that successful sectioning depends very largely on the final consistency of the block. Thin sections are more easily cut from a very rigid block. Very tough tissues require a very dense imbedding medium, whereas softer tissue, like brain and material to be cut in thick sections, may be imbedded in a somewhat softer block.

188. Concentration by Evaporation. Imbedding is most conveniently carried out in a paper boat, coated with thin celloidin which is allowed to dry, and then filled with 8 to 16 per cent. celloidin. The tissue is pushed down into the celloidin and orientated roughly (finer orientation is best done by subsequent trimming of the translucent block). If bubbles develop in the celloidin, the paper boat can be transferred for a short period to a vessel containing ether vapour, BUSSE (*Zeit. wiss. Mikr.*, viii, 1892, p. 467). The bubbles quickly rise to the surface. The boat is then transferred to an empty glass receptacle with the lid adjusted so that very slow evaporation and concentration of the celloidin may proceed. The process appears to involve simply a precipitation of the celloidin owing to the loss of ether from the solvent. Lee recommends that the evaporation should take place under a bell-jar in an atmosphere of alcohol vapour. This is especially indicated for large blocks. After a time the celloidin shrinks down and more thick solution must be added if the surface of the tissue tends to become exposed. When the celloidin is sufficiently hardened the paper can be stripped off to allow even evaporation on all sides of the block. If the " setting " is slow, remove the lid from the container and displace some of the vapour by blowing air into the vessel and then replace the lid. The whole process of adding fresh celloidin and placing the objects under the required conditions of evaporation is repeated every few hours and may be continued for several days if necessary.

APÁTHY (*Zeit. wiss. Mikr.*, v, 1888, p. 47) arranges objects on a small rectangular plate of gelatin, when orientation is difficult. The gelatin plate is placed on the bottom of the imbedding vessel. It is turned out with the celloidin mass after hardening and cut

with it. See also HALLE and BORN (*Zeit. wiss. Mikr.*, xii, 1896, p. 364).

189. Concentration by Heating. Increased concentration of the celloidin, above 16 per cent., can be obtained by infiltrating by the hot method up to 16 per cent. and then adding a few chips of dry celloidin, replacing the cork and incubating until the new celloidin is dissolved. The solution then becomes very viscous but should not be allowed to become solid. The tissue can be removed from the bottle with a spoon or spatula, taking a liberal amount of celloidin with it to form the block. If the celloidin is moulded with the fingers and the tissue pushed into the centre of the mass, it needs only a short exposure to the air before it sets on the surface and is ready for the hardening process (see next section). This rapid imbedding method is very suitable for small pieces of tissue and particularly for tough tissues like tendon where a very dense block is essential.

190. Hardening of the Solid Celloidin Block. Celloidin hardens when placed in reagents such as alcohol, chloroform, benzol and cedarwood oil.

(*a*) Alcohol hardening. The older method of placing the celloidin block, after evaporation of the solvent, into 70 per cent. or 80 per cent. alcohol for one day to several weeks, has been largely superseded by the chloroform hardening first introduced by VIALLANES (*Rech. sur l'Hist. et le Dév. des Insectes*, 1883, p. 129).

(*b*) Chloroform hardening. The block is placed in a large receptacle or a desiccator containing sufficient chloroform to saturate the air in the vessel. The block is allowed to remain overnight or longer and is then immersed in pure chloroform until it sinks. Celloidin blocks prepared by heat concentration (§ 189) need not be placed in chloroform vapour but transferred direct to pure chloroform. It is usually sufficient for thick sections of soft tissues to imbed in 16 per cent. celloidin and, without allowing evaporation of the solvent, to transfer the paper boat to chloroform vapour. Additional hardening of these blocks may be obtained by paraffin wax infiltration. Gilson's mixture of equal parts of chloroform and cedarwood oil (1892) can also be used for final hardening and, after twenty-four hours in this mixture, the blocks are immersed in pure cedarwood oil for several days (see § 195).

(*c*) Paraffin wax hardening. A further variation of the hardening process is generally described as the celloidin-paraffin imbedding technique, although the paraffin wax is used simply to make the solid celloidin block more rigid. The celloidin block, hardened in chloroform vapour and then in pure chloroform, is placed in benzol until it is transparent. It is then infiltrated until saturated with a low melting-point paraffin wax at a temperature not above 38° C. A suitable low melting wax mixture can be made by

adding a small proportion of medicinal paraffin to 45° C. wax. The block is finally infiltrated with 45° C. wax. Care must be taken with the first infiltration to prevent shrinkage of the celloidin block by too rapid removal of the clearing fluid. Small blocks can be transferred direct from chloroform to the low melting-point wax.

191. Rapid Celloidin Imbedding. In certain special instances with very small pieces of material a much more rapid imbedding may be obtained by Gilson's process (1892). The material, after dehydration and soaking in the celloidin solvent, is placed in a thin celloidin solution in a test-tube which is dipped in a bath of melted paraffin. The celloidin is allowed to boil until it becomes viscous. The tissue is then cast in the concentrated celloidin or turned out on to a block of hardened celloidin and the whole hardened in chloroform or a mixture of chloroform and cedarwood oil for one to several hours. The hardened block is then cleared in cedarwood oil, fixed to a block holder and cut with a knife moistened with the oil.

192. Storage of Celloidin Blocks. Celloidin blocks are usually stored in 70 per cent. alcohol or in a mixture of alcohol and glycerin. The blocks infiltrated with cedarwood oil may be kept in air-tight containers, see WALLS (*Stain Tech.*, xi, 1936, p. 89). It is best to cast the paraffin infiltrated blocks in a fresh coating of wax to prevent evaporation of any volatile reagents which may have remained in the celloidin as the result of incomplete infiltration. This applies particularly when the surface of the tissue has been exposed in cutting.

Some histologists prefer to leave the decalcification of bone and teeth until the material is imbedded in celloidin and transferred to alcohol. A radiograph can be taken of the celloidin block beforehand to indicate the distribution and density of the calcification and then the block is immersed in the decalcifying fluid. By taking further radiographs at intervals the material need not be left in the acid for any longer period than is required to obtain complete decalcification. In this way the harmful effects of prolonged exposure of tissues to acids and the objectionable methods of prodding the tissue with a needle to gauge the progress of the decalcification, may be avoided; see HALLPIKE (*Journ. Path. and Bact.*, xxxviii, 1934, p. 249).

193. Mounting and Trimming for Section Cutting. Block holders for celloidin work are now usually made from fibre or hard wood. It is convenient to have a large number of them prepared so that mounted blocks may be stored, pending further section cutting, without detaching them from their holders. The celloidin block is easily attached to a holder by cutting a plane surface on the base of the block and brushing the surface and that of the holder with alcohol-ether. A drop of 16 per cent. celloidin is

placed on the block holder and the block pressed firmly into position. The whole is then placed in chloroform vapour or in alcohol until the fresh celloidin has set. Celloidin blocks infiltrated with paraffin can be attached with hard paraffin wax, provided the wax is well heaped up around the sides of the block. When mounted, the celloidin block is trimmed so that not more than a few millimetres of celloidin are left around the margins of the tissue. It is not essential to have a block with parallel edges and an unnecessarily large margin of celloidin around the section will make flattening difficult when the section is mounted finally on a glass slide. A little more celloidin should be left on the edge of the block, which first meets the knife edge, to accommodate the manipulation of unrolling and flattening the section as it is cut.

194. Section Cutting. It is essential to use a sliding microtome. Celloidin offers more resistance to the knife than paraffin wax and hence the block and the block holder must be very rigidly mounted in a heavily built carrier. The angle of adjustment of the knife depends largely on the size of the block and the nature of the tissue. The long axis of the knife must be inclined at an angle between 45° and 70° to the edge of the block which first meets the knife. The blade of the knife is generally inclined fairly steeply to the upper surface of the block, otherwise the cutting edge will tend to jump through and leave an incomplete section.

(*a*) *Wet cutting method.* For celloidin blocks stored in alcohol and for paraffin-infiltrated blocks, the sections are more easily cut with a knife wet with 70 per cent. alcohol. The knife must be free from grease so that the alcohol will spread evenly over its entire surface. The blade is kept flooded with alcohol during the section cutting (some microtomes are provided with a reservoir adjusted so that the alcohol falls on the blade in drops at intervals). The upper surface of the block is also kept wet with alcohol.

The manipulation required in cutting the sections is not difficult if the knife is sharp and the block is sufficiently rigid. Begin by sliding the knife along towards the block until it just cuts a few millimetres into the corner of the block. Sections thinner than 15μ will generally commence to roll up. The motion of the blade should then be stopped for a moment and the edge of the section unrolled and pressed flat on to the surface of the wet knife with a stiff camel hair brush dipped in alcohol. Then, if the cutting is resumed and the brush is held lightly on top of the section, the latter should slide evenly over the wet surface of the blade. The section can be removed from the knife immediately by fine forceps but generally it is better to slide each section along the blade, arranging them in series if required. When there

is no further room on the knife, the sections can be transferred to a vessel containing 70 per cent. alcohol or placed in series on strips of thin paper moistened with alcohol. It will be found more convenient to continue cutting the sections as quickly as possible and not to hesitate too long between each section. Undue evaporation of alcohol from the block may lead to slight shrinkage and to sections of uneven thickness.

(*b*) *Dry cutting method.* Celloidin blocks infiltrated with cedarwood oil or a similar clearing fluid may be cut with a dry knife. WALLS (*Stain Tech.*, xi, 1936, p. 89) describes a method for cutting dry celloidin sections with a rotary microtome. Some workers prefer to cut paraffin infiltrated blocks with a dry knife but it is doubtful whether very thin sections can be obtained so easily as with the wet method.

195. Preparation of Sections for Staining and Mounting. If sections are to be stained but not kept in serial order, it is usually more convenient to handle them separately and leave the final mounting until after dehydration and clearing. Sections containing paraffin wax must first be dehydrated with absolute alcohol containing 25 per cent., or more, of chloroform and transferred to xylol or benzol to remove the wax; then returned to the absolute alcohol-chloroform and through graded alcohols to water for staining. Small sections can be handled very conveniently if the solutions are placed in small china egg-cups or in small glass staining dishes. Large sections need more careful handling. The method of attaching sections in series to the outer surface of a cylindrical glass bottle by means of rubber bands is to be recommended, see HALLPIKE (*Journ. Path. and Bact.*, xxxviii, 1934, p. 247). The bottle holding three or four circumferential rows of sections can be then immersed on end in glass vessels of slightly larger diameter, containing the staining solutions.

196. Tissues imbedded in celloidin generally show increased affinity for dyes and it will be necessary in many cases to modify the staining techniques originally devised for paraffin sections. Very weak solutions of nuclear dyes, such as Ehrlich's hæmatoxylin, give sharper staining than the full strength solutions, *e.g.* :—

Ehrlich's hæmatoxylin .	0·25 c.c.
Dist. water . . .	50 c.c.

Stain for several hours or overnight.

The common criticism that even thin celloidin sections lack the sharp resolution of paraffin sections, is often due to misuse of the staining solutions.

After staining, the sections are dehydrated with the absolute alcohol-chloroform and transferred to a clearing agent which will soften the celloidin without actually dissolving it. Xylol, toluol and benzol harden the celloidin and prevent even flattening in

Canada balsam. The following oil mixture is to be recommended for routine work :—

Creosote . . .	40 c.c.
Bergamot oil . .	30 c.c.
Xylol	20 c.c.
Origanum oil . .	10 c.c.

The sections are transferred from the oil to clean slides and the major folds straightened out with mounted needles. It may be necessary to use a dissecting microscope for thin sections. A drop of Canada balsam is placed on the section and the coverslip pressed down very firmly. In some cases a small lead weight must be placed on top of the coverslip and allowed to remain overnight. Dunham's mixture :—

White oil of thyme . .	3 to 4 parts
Clove oil	1 part

also serves the purpose of clearing and softening the celloidin. The older clearing fluids containing carbolic acid should be avoided owing to their deleterious action on anilin dyes.

OTHER COLD MASSES

197. Lead-Gum Imbedding Method of J. SALKIND (*C. R. Soc. de Biol.*, lxxix, 1916, p. 16). The principle of the method is that an aqueous solution of gum treated by acetate of lead, when exposed to the action of ammonia, is transformed into a gel, sufficiently stiff to allow of thin sections being cut.

1. Dissolve a quantity of gum of cherry (white for preference) in double its weight of *aq. dest.* After filtration, add to the solution one-third its volume of the liquid subacetate of lead (extract of Saturne), to which has been added 5 per cent. of glacial acetic acid. This gives a kind of thin collodion-like solution, in which you place the pieces at room temperature, to be imbedded, after a fixation, for which see below.

2. Leave about twelve hours for pieces about a millimetre in thickness : larger pieces must be left longer. After the correct period has elapsed, you let the lead-gum solution evaporate in the air till the solution reaches the consistency of a thick celloidin solution.

3. Arrange the pieces to be imbedded in a paper box (or on a piece of paper), in a large drop of the thick solution. Expose to strong ammonia vapour for about five minutes till the block hardens to the consistency of cartilage.

4. Trim the block, and fasten it on to the plate of a microtome by means of some of the thick lead-gum (hardened afterwards in ammonia vapour). Cut sections with an oblique knife, the block being moistened with a solution 1 per cent. sodium chloride

in *aq. dest.* The sections are placed in the same solution, in which they must not stay more than one hour.

5. Sticking the sections to the slide is done by a modification of OLT's method. Cover the slide with albumen, then with gelatin, arrange the sections, press down with a cloth, and harden in formol vapour. See also J. A. Murray, below.

6. The lead-gum is then dissolved away in 5 per cent. acetic acid. After washing you stain and mount in any way desired.

Neither gum arabic (acacia), plum, nor apricot give quite such good results as cherry gum. SALKIND recommends two fixatives to precede this method of imbedding.

A. Formol, acetic acid, sub-acetate of lead, 1 part each. *Aq. dest.*, 5 parts.

B.	Formol	10 c.c.
	Acetone	30 ,,
	Water	40 ,,
	Citric acid	5 to 10 grm.
	Saturated with Sudan III.	

After A, it is not necessary to wash out. After B, and the majority of such fixatives as bichromate especially, you must wash out in running water.

J. A. MURRAY (*Report of Imper. Cancer Research Fund*, 1919) fixes cartilage in 10 per cent. formol-salt solution for at least twenty-four hours. After Salkind's lead-gum imbedding, cuts sections 10—15 μ thick with sliding microtome. Transfers sections for from ten minutes to one hour in 1 per cent. NaCl solution. Special slides prepared beforehand by coating in 1 per cent. gelatin and allowing to dry. See § 227.

The prepared slide is immersed in the salt solution (NaCl), sections arranged with a smooth-pointed glass rod, superfluous liquid drained off, and a wetted cigarette paper carefully lowered over the sections. Firm pressure with several layers of filter paper makes the sections adhere to the slide. Withdraw the cigarette paper and expose to formol vapour for a few minutes. Transfer to 10 per cent. formol five minutes, then treat in the 5 per cent. acetic to remove the lead-gum. Stain.

198. Joliet's Gum and Glycerin Method (*Arch. Zool. Exper. et Gén.*, x, 1882, p. 43). Pure gum arabic dissolved in water to the consistency of a thick syrup. Pour a little of the solution into a watch-glass, and add from 6 to 10 drops of pure glycerin. In the winter or in rainy weather less glycerin should be taken than in the summer or dry weather.

The object is imbedded in the mass in the watch-glass and the whole left to dry for from one to four days. When it has assumed a cartilaginous consistency, a block containing the object is cut out, turned over, and allowed to dry again until wanted for use. A stove, or the sun, may be employed for drying, but it is best to dry slowly at the normal temperature.

199. STRICKER'S **Gum Method** (*Hdb. d. Gewebel.*, p. xxiv). A concentrated solution of gum arabic. The object is imbedded in the gum in a paper case. The whole is thrown into alcohol, and after two or three days may be cut. The alcohol should be of about 80 per cent. (MAYER).

We have seen masses of sufficiently good consistency prepared by this simple method.

200. HYATT'S **Shellac Method**, see *Am. Mic. Journ.*, i, 1880, p. 8; *Journ. Roy. Mic. Soc.*, iii, 1880, p. 320. For sections through hard chitinous organs consisting of several pieces, such as stings and ovipositors, retaining all the parts in their natural positions.

201. BRUNOTTI'S **Cold Gelatin Mass** has been given, § 180.

MASSES FOR GRINDING SECTIONS *

202. G. VON KOCH'S **Copal Method** (*Zool. Anz.*, i, 1878, p. 36). Small pieces of the object are stained in bulk and dehydrated with alcohol. A thin solution of copal in chloroform is prepared by triturating small fragments of copal in a mortar with fine sand, pouring on chloroform to the powder thus obtained and filtering. The objects are brought into a capsule filled with the copal solution. The solution is now slowly evaporated by gently heating the capsule on a tile by means of a common night-light placed beneath it. As soon as the solution is so far concentrated as to draw out into threads that are brittle after cooling, the objects are removed from the capsule and placed to dry for a few days on the tile in order that they may more quickly become hard. When they have attained such a degree of hardness that they cannot be indented by a finger-nail, sections are cut from them by means of a fine saw. The sections are rubbed down even and smooth on one side with a hone, and cemented, with this side downwards, to a slide, by means either of Canada balsam or copal solution. The slide is put away for a few days more on the warmed tile. As soon as the cement is perfectly hard the sections are rubbed down on a grindstone, and then on a bone, to the requisite thinness and polish, washed with water, and mounted in balsam.

The process may be varied by imbedding the objects unstained, removing the copal from the sections by soaking in chloroform, decalcifying them if necessary, and then staining.

It is sometimes a good plan, after removing the copal, to cement a section to a slide by means of hard Canada balsam, then decalcify cautiously the exposed half of the specimen, wash, and stain it.

This method was invented in order to enable the hard and soft parts of corals to be studied in their natural relations, and is valuable for this and similar purposes.

203. EHRENBAUM'S **Colophonium and Wax Method** (*Zeit. wiss. Mik.*, 1884, p. 414). Ehrenbaum recommends a mass consisting of 10 parts of colophonium to 1 of wax. The addition of wax makes the mass less brittle. Sections are obtained by grinding in the usual way. The mass is removed from them by means of turpentine followed by chloroform.

* For the manipulations of section-grinding, see CARPENTER'S *The Microscope*, and §§ 910 *et seq.*

204. JOHNSTONE-LAVIS and VOSMAER'S **Balsam Method** (*Journ. Roy. Mic. Soc.*, 1887, p. 200). Alcohol material is carefully and gradually saturated, first with benzol, and then with thin and thick solutions of benzol-balsam. It is then dried for a day in the air and for several days more in a hot-air bath. When hard it is ground in the usual way.

205. WEIL'S **Canada Balsam Method**, see *Zeit. wiss. Mik.*, v, 1888, p. 200.

206. GIESBRECHT'S **Shellac Method.** For hard parts only, spines of *Echinus*, shell, etc., see *Morph. Jahrb.*, vi, 1880, p. 95, or the abstract in LEE und MAYER, *Grundzüge*.

CHAPTER XI

SERIAL SECTION MOUNTING

207. Choice of a Method. We recommend the following :—For *general* work with paraffin sections, *the combined water and albumen* method, § 210. For very delicate work, *the water method.* For collodion sections, the *albumen method ;* for large collodion sections, GRAHAM KERR's seems the most convenient.

METHODS FOR PARAFFIN SECTIONS

208. The Water or Desiccation Method. GAULE (*Arch. Anat. Phys., Phys. Abth.*, 1881, p. 156); SUCHANNEK (*Zeit. wiss. Mik.*, vii, 1891, p. 464); GULLAND (*Journ. Anat. and Phys.*, xxvi, 1891, p. 56); SCHIEFFERDECKER (*Zeit. wiss. Mik.*, ix, 1892, p. 202); HEIDENHAIN (*Kern. und Protoplasma*, p. 114); NUSBAUM (*Anat. Anz.*, xii, 2, 1896, p. 52); MAYER in the *Grundzüge*, LEE und MAYER, 1898, p. 113; DE GROOT (*Zeit. wiss. Mik.*, xv, 1898, p. 62), and others. The principle of this method is that the sections are made to adhere to the slide without the intervention of any cementing substance, being brought into intimate contact with the glass by being slowly drawn down by the evaporation of a layer of water on which they are floated. It is now practised, with unessential variations, as follows :

(*a*) For sections that are *large and not numerous*. The sections are flattened out on water by one or other of the processes described in § 169. The slide is then drained and put away to dry until every trace of water has completely evaporated away from under the sections. This drying may be performed at the temperature of the laboratory, in which case many hours will be necessary (to be safe it will generally be necessary to leave the sections overnight). Or it may be performed in a stove or on a water-bath at a temperature a few degrees *below* the melting-point of the paraffin (best not above 40° C.), in which case fixation will be much more rapid, large thin sections being often sufficiently fixed in an hour, though thick ones will require half a dozen hours or more. *The paraffin must not be allowed to melt before the sections are perfectly dry ;* the sections are sure to become detached if it does. Perfectly dry sections have a certain brilliant transparent look that is easily recognisable. As soon as dry the paraffin may be removed, and they may be further treated as desired. To remove the paraffin all that is requisite is to put the slide in a

tube of xylol or other good solvent, which in a few seconds, or minutes at most, removes the paraffin perfectly. Most workers first melt the paraffin, but Lee found this is not necessary.

(*b*) For series of *numerous small sections.* Clean a slide perfectly, so that water will spread on it without any tendency to run into drops (see below). Breathe on it, and with a brush draw on it a streak of water as wide as the sections and a little longer than the first row of sections that it is intended to mount. With a dry brush arrange the first row of sections (which may be either loose ones or a length of a ribbon) on this streak. Breathe on the slide again, draw on it another streak of water under the first one and arrange the next row of sections on it, and so on until the slide is full. Then breathe on the slide again, and with the brush add a drop of water at each end of each row of sections, so as to enable them to expand freely; then warm the slide so as to flatten out the sections, taking care *not to melt the paraffin*. Some persons do this by holding it over a small flame for a few seconds. Lee preferred to lay it on a slab of thick glass, warmed, watching the flattening of the sections through a lens if necessary. As soon as they are perfectly flat, draw off the excess of water from one corner of the mount with a dry brush, and put aside to dry as before (*a*).

In order to succeed in this method it is absolutely essential that the sections be perfectly expanded and come into close contact with the slide at all points. And to ensure this it is necessary that the slide should be perfectly *free from grease*, so that the water may wet it equally everywhere. The test for this is, firstly, to breathe on the slide; the moisture from the breath should condense on it evenly all over, and disappear evenly. Secondly, streaks of water drawn on it with a brush should not run. To obtain a slide that will fulfil these conditions, clean it well in the usual way, place a drop of water on it and rub it in thoroughly with a damp cloth and try the tests. If this does not suffice, take a turn of a corner of the cloth round a finger and rub it with a piece of chalk, then damp the cloth and rub the slide with it, finishing up with a clean part of the cloth and clean water (De Groot, *loc. cit.*, *supra*). If after performing this operation twice the slide still refuses to take the water thoroughly it should be rejected as incorrigible; for there are apparently some sorts of glass that can never be got to wet properly. Mayer finds carbonate of magnesia or soda useful.

Gudernatsch (*Zeit. wiss. Mikr.*, xxiv, 1908, p. 358) washes the slide well with potash soap, and arranges the sections on it whilst still wet. Helly (*ibid.*, 1906, p. 330) passes it two or three times over the flame of a Bunsen burner.

Tap water seems preferable to distilled water; it seems to spread better and to give a stronger adhesion. Nusbaum adds a trace of gum

arabic (1 or 2 drops of mucilage to a glass of water); Apáthy (*Microtechnik*, p. 126) adds 1 per cent. of Mayer's albumen (§ 209); and Henneguy (*Leçons sur la Cellule*, 1896, p. 62) takes a 1 : 5,000 solution of gelatin, with a trace of bichromate of potash, added just before using, and dries the slides exposed to light. Similarly, Burchardt (*Jena Zeit.*, xxxiv, 1900, p. 719).

Some workers have used alcohol (50 or 70 per cent.) instead of water; but this we believe to be now generally abandoned.

This is the most elegant method of any, as there is nothing on the slide except the sections that can stain, or appear as *dirt in the mount.* Tissues do not suffer from the drying, provided the material has been properly imbedded. Sections stick so fast by this method that they will stand watery or other fluids for weeks, *so long as they are not alkaline.* When successfully performed it is quite safe, provided that the sections are *of a suitable nature.* They must be such as to afford a sufficiently *continuous surface*, everywhere in contact with the slide. Sections of parenchymatous organs stick well; sections of thin-walled tubular organs stick badly. Sections of chitinous organs are very unsafe. The larger and *thinner* sections are, the better do they stick, and *vice versâ.* Sections from chromic or osmic material adhere less well than sections from alcohol or sublimate material.

By taking a staining solution instead of pure water for expanding, the sections can be got to stain at the same time, and so be brought into balsam without passing through alcohol; see Mayer, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 320; Schmorl, *Path.-hist Untersuchungsmethoden*, 1897, p. 38; Smith, *Journ. Anat. Phys.*, xxxiv, 1899, p. 151.

209. Mayer's **Albumen** (*Mitth. Zool. Stat. Neapel*, iv, 1883; *Internat. Monatschr. f. Anat.*, iv, 1887, p. 42). White of egg, 50 c.c.; glycerin, 50 c.c.; salicylate of soda, 1 grm. Shake them well together, and filter into a clean bottle. The filtering may take days or a week, but the preparation does not spoil meanwhile.

Francotte shakes up the albumen with a few drops of acetic acid before adding the other ingredients, and finds the filtering greatly quickened.

A *very thin* layer of the mixture is spread on a slide with a fine brush and well rubbed in with the finger (Lee preferred a small rubber "squeegee"). The sections are laid on it and pressed down lightly with a brush (if they will bear it). The slide may then be warmed for some minutes on a water-bath, and the paraffin removed with a solvent.

It is *not necessary* to warm the slide at all; *the paraffin can be removed in the cold if desired* by putting the slide into toluol, xylol, or the like. But the slide *must*, in any case, be *treated with alcohol* after removal of the paraffin, in order to get rid of the glycerin, which will cause cloudiness if not perfectly removed.

This method allows of the staining of sections on the slide with perfect safety, both with alcoholic and aqueous stains, provided they be *not alkaline.*

According to LEE's experience, the albumen method is *absolutely safe*, provided that *alkaline fluids be avoided* in the after-treatment. It has the defect that certain plasma stains (not chromatin stains) colour the albumen very strongly, and cannot be removed from it, and that sections are not expanded by it.

It sometimes happens that the mixture after it has stood for some time becomes turbid, and at last coagulates, passing into a caseous state; or it may undergo a hyaline coagulation, drying up like amber. But up to the very last it does not *in general* lose its adhesive properties. We have, however, found it to do so, after keeping for five or six years, so that, to be on the safe side, it may be well to make it up fresh every six months.

HEIDENHAIN (*Zeit. wiss. Mikr.*, xxii, 1905, p. 331) makes it up with 1 grm. of blood albumen dissolved in 25 c.c. of water, and an equal volume of 50 per cent. alcohol.

210. The Albumen and Water Method (HENNEGUY, *Journ. de l'Anat. et de la Physiol.*, 1891, p. 398). A drop of water is spread on a slide painted with Mayer's white-of-egg mixture, the sections are arranged on it, the whole is warmed (*not* to the melting-point of the paraffin) until the sections flatten out; the water is then evaporated off at a temperature of about 40° C., and as soon as it has sufficiently disappeared, which at that temperature will be in about *ten to fifteen minutes*, the slide is further treated as described last §.

This is a most valuable method. It is quicker than the water method, and, for difficult material, safer.

See also OHLMACHER, *Journ. Amer. Med. Assoc.*, April, 1893.

The so-called "Japanese" method, attributed to IKEDA by REINKE (*Zeit. wiss. Mik.*, xii, 1895, p. 21), *is merely that of* HENNEGUY.

MANN (*Anat. Anz.*, viii, 1893, p. 442) shakes up white of egg with water, coats slides with it and dries them. He flattens sections on water at 40° C., lifts them out on a prepared slide, and dries for five minutes at 35° C.

211. Garlic-water. HOLLANDE (*Arch. d'Anat. Micr.*, xiii, 1911, p. 171) gives the following as more adhesive than albumen :—50 grm. of crushed and chopped garlic are rubbed up with 80 c.c. of chloroform-water (Codex, A.C.) and filtered after twenty-four hours. Use as albumen.

212. SCHALLIBAUM's **Collodion** (*Arch. mikr. Anat.*, xxii, 1883, p. 565). One part of collodion shaken up with 3—4 parts of clove or lavender oil. Use as albumen. Sections can be treated with alcohol (not absolute) and divers staining fluids. Lee did not find it safe for this. RABL, however (*Zeit. wiss. Mik.*, xi, 1894, p. 170), finds that it is if you take 2 parts of collodion to 3 of clove oil, and make up fresh every four or five days.

213. OBREGIA's **Method for Paraffin or Celloidin Sections** (*Neurologisches Centralb.*, ix, 1890, p. 295; GULLAND, *Journ. of Path.*,

February, 1893). Slides, or glass plates of any size, are coated with a solution made of—

Syrupy solution of powdered candy-sugar made with boiling distilled water .	30 c.c.
95 per cent. alcohol	20 ,,
Transparent syrupy solution of pure dextrin made by boiling with distilled water	10 ,,

They are dried slowly for two or three days until the surface is just sticky to the moist finger. *Paraffin sections* are arranged and heated for a few minutes to a temperature slightly above the melting-point of the paraffin. The paraffin is removed by some solvent, and this in turn by absolute alcohol. The alcohol is poured off, and the sections are covered with solution of celloidin. The plates are left to evaporate for ten minutes in a horizontal position, then brought into water, in which the sheet of celloidin with the sections soon becomes detached, and may be further treated as desired, *e.g.* as in Weigert's process, § 220. The evaporation must not be artificially hastened.

DIMMER (*Zeit. wiss. Mik.*, xvi, 1899, p. 44) coats the slides with a solution of about 16 parts of gelatin in 300 of warm water, and dries them (two days), and proceeds in other respects as above.

A good method for *large* sections, equally applicable to paraffin sections, to celloidin sections, and to sections of material that has not been imbedded at all.

For BLOCHMAN's modification of Weigert's process, by means of which large sections can be preserved *unmounted*, see *Zeit. wiss. Mik.*, xiv, 1897, p. 189.

214. STRASSER's **Collodion Paper Method** (*ibid.*, iii, 1886, p. 346). This is an extremely complicated modification of Weigert's method for celloidin sections, and is only adapted for use with STRASSER's automatic ribbon-microtome. See *Zeit. wiss. Mik.*, iii, 1886, p. 346; vi, 1889, p. 154; vii, 1890, pp. 290 and 304; ix, 1892, p. 8; xii, 1895, p. 154; and xiv, 1897, p. 39; also SCHOENEMANN, *ibid.*, xix, 1903, p. 333; STRASSER, *ibid.*, p. 337; and RUPPRICHT, *ibid.*, xxviii, 1912, p. 281.

METHODS FOR WATERY SECTIONS

215. FOL's **Gelatin** (FOL, *Lehrb.*, p. 132). Four grammes of gelatin are dissolved in 20 c.c. of glacial acetic acid by heating on a water-bath and agitation. To 5 c.c. of the solution add 70 c.c. of 70 per cent. alcohol and 1 to 2 c.c. of 5 per cent. aqueous solution of chrome-alum. Pour the mixture on to the slide and allow it to dry. In a few hours the gelatin passes into the insoluble state. It retains, however, the property of swelling and becoming somewhat sticky in presence of water. The slide may then be immersed in water containing the sections; these can be slid into their places, and the whole lifted out; the sections will be found to be fixed.

This method is specially intended for sections made under water, *large celloidin sections* amongst others.

Similarly, RUPPRICHT, *loc. cit.*, last §, with the needless complication of a seriation on Strasser's collodionised paper.

STRASSER (*loc. cit.*, last §) also employs a dry gelatin film which he makes sticky by means of carbol-xylol.

METHODS FOR CELLOIDIN SECTIONS

216. The Albumen Method. Lee finds that celloidin sections may be mounted on Mayer's albumen, and have the celloidin removed, if desired, by putting them into ether-alcohol. Care must be taken to press them down very thoroughly on to the albumen; and it is well not to have them too wet.

Similarly, JORDAN (*Zeit. wiss. Mik.*, xv, 1898, p. 54), and ARGUTINSKY (*ibid.*, xvii, 1900, p. 37). See also JORDAN, *ibid.*, 192—194; DANTSCHAKOFF, *ibid.*, xxv, 1908, p. 35; MAXIMOW, *ibid.*, xxvi, 1909, p. 184; ANITSCHKOW, *ibid.*, xxvii, 1910, p. 68; WEBER, *ibid.*, xxix, 1912, p. 186; RUBASCHKIN, *Anat. Anz.*, xxxi, 1907, p. 30. Weber paints over the series on the albumen with a layer of thin collodion, and puts into alcohol of 50 per cent., then into a mixture of equal parts of chloroform and absolute alcohol. After staining, pure absolute alcohol must be avoided.

217. SUMMERS' **Ether Method** (*Amer. Mon. Mic. Journ.*, 1887, p. 73). Place the sections in 95 per cent. alcohol for a minute or two, arrange on the slide, and then pour over the sections sulphuric ether *vapour*, from a bottle partly full of liquid ether. The celloidin will immediately soften and become perfectly transparent. Place the slide in 80 per cent. alcohol, or even directly in 95 per cent. if desired. We have not ourselves found this method safe.

Instead of pouring the ether vapour over the slide, it may, of course, be treated with ether vapour *in a preparation glass or similar arrangement*, which Lee thinks preferable.

GAGE (*Proc. Amer. Soc. Mic.*, 1892, p. 82) advises that the slide be one that has been previously coated with a 0·5 per cent. solution of white of egg and dried; the collodion adheres much more strongly to an albuminised surface.

AUBURTIN (*Anat. Anz.*, xiii, 1897, p. 90) arranges on a clean slide, dehydrates the sections with blotting-paper and treatment with absolute alcohol, then drops on to them a mixture of alcohol and ether which dissolves out the celloidin from the sections, then allows the thin collodion thus formed to evaporate into a thin sheet on the slide. Then 70 per cent. alcohol and other desired reagents.

Similarly, MAIER (*Munch. med. Wochenschr.*, lvii, 1910, No. 12; *Zeit. wiss. Mik.*, xxvii, 1910, p. 385), but adding a treatment for ten to fifteen minutes with bisulphide of carbon.

See also MYERS, *Arch. Anat. Phys., Anat., Abth.*, 1902, p. 371 (complicated).

218. APÁTHY'S **Oil of Bergamot Method** (*Mitth. Zool. Stat. Neapel*, 1887, p. 742; *Zeit. wiss. Mik.*, v, 1888, pp. 46 and 360, and vi, 1889, p. 167). Cut with a knife smeared with yellow vaseline and wetted with 95 per cent. alcohol. Float the sections,

as cut, on bergamot oil (must be green, must mix perfectly with 90 per cent. alcohol, and must not smell of turpentine), or on carbolxylol (*Mikrotechnik*, p. 176). The sections *flatten themselves out* on the surface of the oil, and are then transferred to a slide which (APÁTHY, *Mikrotechnik*, pp. 127 and 176) has been previously collodionised and dried.

If the sections are to be stained, the slide after removal of the bergamot oil, by a cigarette paper, is exposed for a few minutes to the vapour of a mixture of ether and alcohol, then brought into 90 per cent. alcohol, and after a quarter of an hour therein may be stained in any fluid that contains 70 per cent. alcohol or more.

If it be desired to stain in a watery fluid, care must have been taken when arranging the sections to let the celloidin of each section overlap that of its neighbours at the edges, so that the ether vapour may fuse them all into one continuous plate. This will become detached from the slide in watery fluids, and may then be treated as a single section. Terpinol may be taken instead of bergamot oil.

219. APÁTHY'S **Series-on-the-Knife Method** (*Zeit. wiss. Mik.*, vi, 1888, p. 168). The knife is well smeared with vaseline, rubbed evenly on, and is wetted with alcohol of 70 to 90 per cent. As fast as the sections are cut they are drawn with a needle or small brush to a dry part of the blade, and there arranged in rows, the celloidin of each section overlapping or at least touching that of its neighbours. When a series (or several series, if you like) has been thus completed, the sections are dried by laying blotting-paper on them, and the series is painted over with some of the thinnest celloidin solution used for imbedding, is allowed to evaporate for five minutes in the air, and the knife is then removed and brought for half an hour into 70 per cent. alcohol. This hardens the celloidin around the sections into a continuous lamella, which can be easily detached by means of a scalpel, and stained, or further treated as desired.

220. WEIGERT'S **Collodion Method** (*Zeit. wiss. Mikr.*, 1885, p. 490). Slides, or larger plates of glass, are prepared by coating them with collodion in a thin layer, as photographers do, and allowing them to dry (they may be kept thus in stock). Sections (cut wet with alcohol) are got on to one of these (by a roundabout process, not essential) and arranged in order, and gently pressed down with paper.

Now remove with blotting-paper any excess of alcohol that may remain on or around the sections, pour collodion over them, and get it to spread in an even layer. As soon as this layer is dry at the surface you may write any necessary indications on it with a small brush charged with methylen blue (the colour will remain fast throughout all subsequent manipulations).

The plate may now be either put away till wanted in 80 per cent. alcohol, or may be brought into a staining fluid. The watery fluid causes the double sheet of collodion to become detached from the glass, holding the sections fast between its folds. It is then easy to stain, wash, dehydrate, and mount in the usual way, merely taking care not to use alcohol of more than 90 to 96 per cent. for dehydration. Weigert recommends for clearing the mixture of xylol and carbolic acid (3 : 1).

The series should be cut into the desired lengths for mounting whilst in the alcohol.

A good method for *large* and *thick* sections.

For Blochman's modification see § 213.

Strasser takes gummed paper instead of the glass plates used in this process. See the papers quoted § 214.

See also Wintersteiner (*Zeit. wiss. Mik.*, x, 1893, p. 316) and Kubo (*Arch. mik. Anat.*, lxx, 1907, p. 173).

221. Obregia's **Method.** Slides are prepared as directed (§ 213), the sections are arranged on them and covered with celloidin or photoxylin and evaporated as described, § 213.

For Dimmer's modification see also § 213.

222. Collodion Film Method. Graham Kerr (*in litt.*, 1908) seriates on *Kodak films.* A film has the emulsion removed by hot water. The sections are arranged on a dry film, and the application of a drop of absolute alcohol and ether (or an atmosphere of alcohol and ether) suffices to weld them into a mass with the film. The sheet may then be stained and mounted or rolled up and stored in cedar oil.

The late Dr. S. G. Scott used mica sheets, upon which he stuck paraffin sections. These could be distributed to a class of students by simply cutting out pieces of mica supporting the sections.

Other Methods for Celloidin Sections. See § 215 (Fol) and § 227 (Olt).

CHAPTER XII

PREPARATION OF SECTIONS BY FREEZING TECHNIQUE

223. The preparation of sections by freezing with CO_2 gas is now a positive and indispensable method used in laboratories where experimental work is carried out, and in pathological and surgical institutions where rapid diagnosis is necessary. This method has a number of important advantages, two of which are that the time ordinarily taken in imbedding in paraffin wax or other masses is saved, and that first contact of tissues with fixing fluids may be avoided. Serial sections cannot be prepared by this method, though with great experience something approaching this may be achieved. The possibilities of the frozen section are not yet realised in zoological laboratories and the method should be of great use to cytologists. In microchemical technique the frozen sectioning microtome is indispensable.

In the older models of this microtome, ether was used for freezing, and it was usual to soak the pieces for some hours in gum, dextrine or sugar solutions to prevent ice crystals forming. In the most modern technique, made possible by the use of CO_2 jets on the microtome knife, the sections are stuck upon the slide by means of their own albuminous (proteid) juices, and no water is used until after the sections have been fixed in osmic or formalin vapour.

It is believed by some workers that frozen sections are not fit for the best cytological study. This is wrong, for sections made by this method and stained by Hollande's chloro-carmine method, for example, can be extremely delicate and beautiful.

224. Freezing Microtomes. A number of firms make admirable microtomes, but, if possible, an instrument with a knife cooling attachment should be purchased. This is necessary for the best type of work.

FROZEN SECTIONS

225. Preparation of Material. Material may be cut fresh or after fixation. In the latter case any of the usual fixatives, such as Zenker, formalin 10 per cent., Bouin, etc., are suitable. For quick surgical diagnosis 40 per cent. formaldehyde is generally used, small pieces of tissue being put in the fluid until they are penetrated and sink. Tissues containing much fat will not sink

and should be left about half an hour. Osmic-fixed tissues get very brittle in the freezing technique.

226. Cutting Sections. The piece to be cut should not ordinarily exceed 3×3 cm. One corner should lie towards the knife, and fibres should, if possible, be at right-angles to the edge of the knife. No water, saline, gum, syrup, etc., should be brought into contact with the tissue pieces. A little water will have to be put on the microtome table freezer in order to freeze the piece of tissue securely on to the table. The indicator for thickness of sections should be placed at 20 μ or thereabouts, when the beginner is practising—it is difficult to cut thinner sections.

According as to whether the sections are to be stuck on to slides with gelatin, etc., or " dry " by their own juices coagulated by some suitable vapour, the procedure is slightly different. The former method is probably the easier, and should be mastered first. In both cases, however, *the temperature of the frozen piece is important and can only be judged by experience.* Material like brain, liver, spleen, etc., should be about $-10°$ to $-15°$ C.; fat, ligament, etc., $-20°$ to $-30°$ C. Turn on the cock on the cylinder of CO_2, open the lever below the freezer, and with several openings and shuttings of the pin valve of the freezer, the block becomes frozen. The knife, which has previously been brought into proper juxtaposition to the block, by raising or lowering the freezer table, is now passed over the tissue till sections begin to cut. Look at these sections—if they have fine cracks on them or are brittle, the block is too cold, if they are torn, the block is not cold enough. A piece of liver is excellent material for judging this degree of freezing, which can only be learnt after some practice. The proper degree of temperature is more easily judged with the microtome fitted with a knife freezing attachment. When cut, ordinary mammalian sections of fixed material may be removed from the knife with a brush or forceps and transferred to a dish of distilled water or 10 per cent. formol salt in which they may remain until stuck on slides as described below. Sections of such materials as mollusc ovotestis and mammalian testis tend to break up if floated out in a dish, and are best cut fresh, if possible, according to the method described in § 230. However, they can be cut and stuck on a slide if brought direct to the gelatin coated slide one at a time.

227. The Gelatined Slides. These are prepared by smearing gelatin on the slide in the same manner as a blood film. About 2 grm. of the best gelatin is broken up and dissolved in 100 c.c. of water as follows: it is first left to soak in enough distilled water to cover it for about two or three hours and then the rest of the 100 c.c. of distilled water is added and heated to 50° or 60° C. Clean slides are coated with this fluid as for making blood smears. The slides are then tilted up against the back of the bench till

they dry, the gelatin surface being inside so as to avoid dust. These slides will dry quickly in a warm place. The gelatin solution does not keep well and it is better to make a supply of slides just for the work in hand. When handling the slides the gelatin side can be discovered by breathing on the slide, the moisture showing on the side without the gelatin. If the gelatin is staining visibly in the finished preparations, you are smearing the slides too thickly when preparing.

228. Miller's Method for Mounting Frozen Sections. E. G. Miller (*J. R. Micr. Soc.*, 1930), who is an expert in the frozen section technique, mounts as follows: a glass rod is drawn out and bent at an angle of 130 degrees, cut not less than an inch from the bend, and the cut end rounded off in the flame. A Petri dish is filled with distilled water and the best section from the other dish containing the sections is selected, and the short limb of the glass rod passed under the section to pick it up. A gelatined slide is now taken in the left hand, plunged into the Petri dish of distilled water, holding it just below and parallel to the surface. Holding the glass rod in the right hand, dip into the water and allow the section to float clear, gently raising the slide out of the water, catching the section in the middle of the slide. Tilt the slide gently, allowing the water to run off. Place the slide on the bench, put a cigarette paper previously wetted on both sides over the section, and press down with a pad formed by a folded filter paper, rubbing one way so as to press without shifting either paper. Remove the pad of filter paper and gently peel off the cigarette paper. If the section is fatty or sticky, it may adhere to the cigarette paper. To remedy, wet paper with a few drops of pyridine in 50 per cent. alcohol. If not already fixed, place the section in a corked specimen tube or staining jar, at the bottom of which is a plug of cotton wool soaked in strong formalin. This jar should be placed on a warm plate, or be warmed so that the formaldehyde gas comes off well. Leave slide fifteen to thirty seconds to fix. Transfer to 10 per cent. formol saline.

229. Staining Frozen Sections. Sections or smears which have just been fixed are difficult to stain. This can be remedied by immersing overnight in 90 per cent. alcohol, after which they will stain evenly. But this may not be desirable and it may be necessary to proceed straight away. The most beautiful method is undoubtedly Hollande's chloro-carmine-iron alum. Some of Mr. E. G. Miller's slides are very fine, showing both chromosomes and mitochondria well. For sections which do not stain well, he recommends leaving overnight in formol-saline. If they do not stain properly, treatment with H_2O_2 should then be tried. Slides should be left in one volume of H_2O_2 to nine volumes of 70 to 90 per cent. alcohol for approximately thirty minutes.

Miller (*op. cit.*) stains usually in Hollande's chloro-carmine-iron alum as follows: Hollande one minute. Rinse 30 per cent. alcohol, then quickly through distilled water to 3·5 per cent. iron alum, till black. Control under microscope. Wash in distilled water, dip in 5 per cent. pyridine in distilled water. Wash in distilled water. Transfer to running tap water for at least ten minutes—then distilled water and upgrade and mount in balsam.

230. For Cytological or Histological work of the best type, the knife cooling attachment should be used. When the section is two-thirds cut, place a dry, clean coverslip, held in a pair of forceps, just where the section is curling up, and it will adhere to the coverslip. By gently pulling knife and section on the coverslip towards you the whole section will thaw on to the surface of the coverslip. The section may now be fixed immediately in osmic or formalin vapour, and then be placed in the fixing fluid desired. With gentle treatment such sections stick well to the coverslips (or slides, if these be preferred) until finally stained and mounted. It may be necessary to spread out the section on the frozen knife with a dry camel-hair brush, before making it adhere to a coverslip or slide, and finally finishing the sweep of the knife. It is difficult to describe this process, but it is fairly easy to master the trick of getting the sections on to the glass after some practice. It should be remembered that sections must be rolled out on the frozen knife and not on the slide, in those cases where they curl badly.

It may be mentioned that the development of this method is due to Messrs. Leitz. It is a definite advance in microtomy.

231. Gelatin Imbedding for Freezing Technique, refer to § 179, which gives the recent method of Zwemer.

232. Glychrogel Mounting Solution of Zwemer for Frozen Sections, refer to § 474.

CHAPTER XIII *

STAINING

233. Dyes. It is hardly the place in such a book as this to go at all deeply into the subject of dye chemistry. There are, nevertheless, certain rather fundamental principles in connection with chemistry which are often useful for the biologist to bear in mind when he is considering what dyes to select for any particular staining procedure. A few of these most fundamental principles of dye chemistry will be discussed here. For more detailed information the reader is referred to CONN ("Biological Stains," 3rd ed., Geneva, N.Y., 1936).

From the standpoint of the biologist, dyes may be classified as follows :—

A. Simple dyes.
- (1) Artificial dyes.
  - (*a*) The nitro dyes (*e.g.* picric acid).
  - (*b*) The azo dyes (*e.g.* orange G, Bordeaux red, the Sudans, Bismarck brown and Congo red).
  - (*c*) The oxyquinone dyes (*e.g.* alizarin).
  - (*d*) The quinone-imide dyes (*e.g.* thionin, methylen blue, the azures, toluidine blue, neutral red and the safranins).
  - (*e*) The phenyl methane dyes (*e.g.* brilliant green, light green, the fuchsins and methyl violets, methyl green and the anilin blues).
  - (*f*) The xanthene dyes (*e.g.* the pyronins, eosin, erythrosin, phloxine and rose Bengal).
- (2) The natural dyes.
  - (*a*) Logwood and brazilwood derivatives.
  - (*b*) Cochineal derivatives.
  - (*c*) Miscellaneous (*e.g.* safranin, indigo, orcein and litmus).

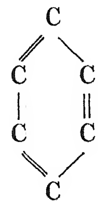
B. Neutral stains or compound dyes (*e.g.* the Romanovsky blood stain and its various modifications).

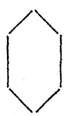
Dyes of any of the above groups except the last may be either acidic or basic, these two terms indicating, as explained below, not the reaction of the dye, but whether the dye property is carried by the anion or the cation. For practical purposes it is often more necessary to know whether a dye is acidic or basic

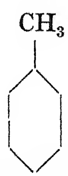
* By H. J. C.

than to place it in one or another of the above-mentioned chemical groups.

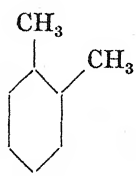
234. The General Nature of Dyes. The artificial dyes are all derivatives of benzene which has the structural formula



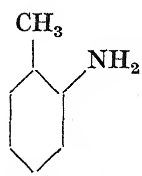
and is ordinarily indicated in more complex formulæ as a mere hexagon thus: . There is a series of derivatives of benzene which is also of importance in the constitution of dyes, among which is anilin . Because so many of the first artificial dyes produced were derived from anilin, the term "anilin dyes" is often used to apply to all these products, although anilin does not enter into the formation of all the synthetic dyes at present manufactured. Other simple derivatives of benzene from which many dyes are derived are the following:—



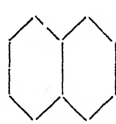
Toluene



Xylene (ortho)



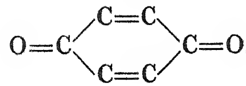
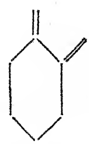
Toluidine (ortho)



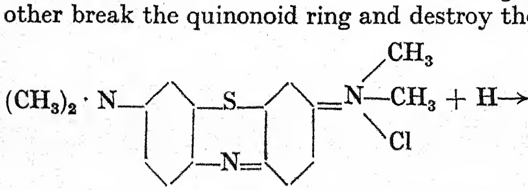
Naphthalene

The last of these, as will be readily understood, is a condensation of two benzene rings sharing two carbon atoms and having only eight hydrogen atoms between them.

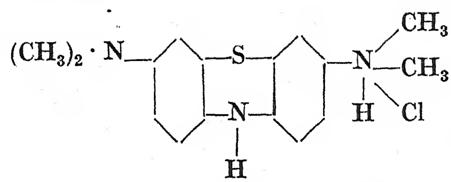
Another simple derivative of benzene which has a very intimate relation to many of the dyes is quinone. This compound is a dioxide of benzene; and to account for the replacement of two monovalent hydrogen atoms by two bivalent oxygen atoms, it is necessary to assume a rearrangement of the internal bonds of the

benzene nucleus. The formula ordinarily assumed for quinone, therefore, is  This formula is more commonly written in the simpler form  In this compound it will be noticed there is a different type of benzene ring from that observed in derivatives like xylene or toluidine, as it has two sets of external double bonds instead of single bonds at each carbon atom; it is known as the quinonoid ring. Two types of quinonoid rings are known, the *para*  and the *ortho* . Ordinarily a compound containing the quinonoid ring is coloured, although not necessarily a dye; and any chemical which, due to reduction, oxidation, or change in reaction, destroys the quinonoid structure, thus returning the normal form of the benzene ring, decolorises the compound. This is important to remember in connection with the colourless forms of such dyes as fuchsin and methylen blue.

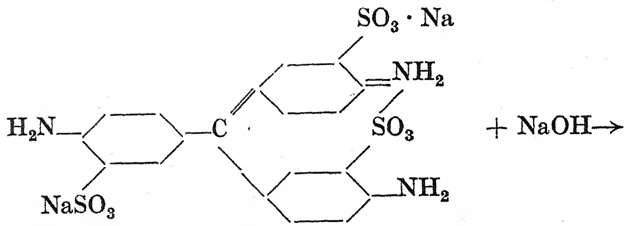
235. Chromophores. Radicals like the quinonoid ring which have the above-mentioned relation to colour in the compounds are known as chromophores. There are quite a number of radicals of this nature known, but only one beside the quinonoid ring is of special significance from the standpoint of the biological stains; namely, the azo group ($-N=N-$), which occurs in the azo dyes. Of all the chromophores the quinonoid ring is by far the most significant and occurs in the greatest variety of dyes. Methylen blue and acid fuchsin are good examples of the importance of this chromophore; the reactions illustrated below show how reduction in one case and change of reaction in the other break the quinonoid ring and destroy the colour.



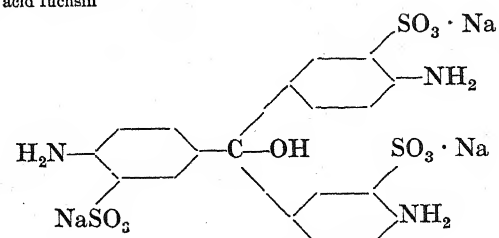
Methylen blue



Leuco-methylen blue (colourless)



Disodium pararosanilin trisulphonate (red)—one of the primary constituents of acid fuchsin



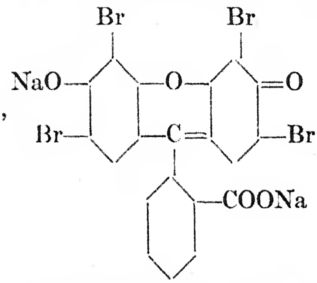
Trisodium pararosanilin trisulphonate (colourless)

236. Salt-forming Radicals. A compound containing a chromophore, although usually coloured, is not necessarily a dye. To become a dye it must contain an additional radical of such a nature as to allow the compound to act either as an acid or a base, and thus to form salts with either metallic bases or mineral acids. Most important of these salt-forming radicals are the amino ($-NH_2$) and hydroxyl ($-OH$) groups, which, because of their peculiar relation to dyes are sometimes called "auxochromes." Two other salt-forming radicals which occur in many dyes are the carboxyl ($-COOH$) and the sulphonic acid groups ($-SO_3H$). An illustration of the latter may be seen in acid fuchsin, as shown above, in which three sulphonic acid groups occur, two of which have combined with sodium to form a salt. Methylen blue illustrates the salt-forming properties of the amino group: it will be seen that in one of the methyl-substituted amino radicals the nitrogen has become pentavalent, enabling the compound to act as an ammonium base and to combine with the mineral acid HCl to form a chloride.

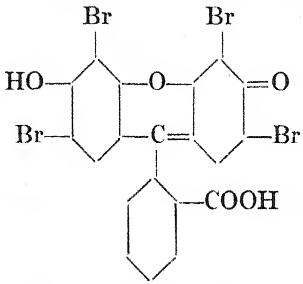
It is primarily these salt-forming radicals which give a dye its acidic or basic character. Thus, the majority of the basic dyes contain the amidogen group and act as ammonium bases to combine with mineral acids like hydrochloric or sulphuric. Basic

dyes, therefore, are salts which owe their coloured properties to the cation, while the anion is that of an inorganic acid (or an organic acid having no coloured properties). Acid dyes, on the other hand, contain an acid radical such as —OH, $—SO_3H$, or —COOH and form salts with strong bases like sodium and potassium, the resulting dyes containing their chromophore group or groups in the anion, not the cation. Basic dyes on the market ordinarily occur as chlorides, occasionally sulphates or acetates. Acid dyes are ordinarily sodium salts, although sometimes salts of potassium or occasionally of some other metal.

237. Colour Acids and Bases. Although the above-mentioned salts are the normal forms in which dyes are available, most acid dyes may be obtained in the form of the colour acids and some basic dyes in the form of the colour bases. Thus the colour acid of eosin,

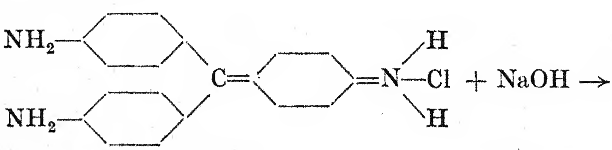


is :

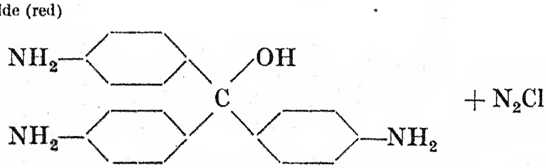


The latter compound is coloured like its salt, as might be inferred from the fact that it retains the quinonoid ring. It is almost insoluble in water, however (nearly all colour acids being alcohol-soluble, but not water-soluble), and has no dye properties except in the presence of alkali when it is converted into one of its salts.

The colour bases of most basic dyes, however, are known in theory only, all efforts to convert such dyes into their bases destroying the quinonoid structure and resulting in the production of so-called pseudo-bases or leuco-bases. Thus pararosanilin, one of the primary constituents of basic fuchsin, yields its leuco-base as follows :—



Pararosanilin chloride (red)

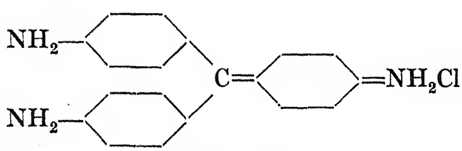


Pararosanilin carbinol (colourless)

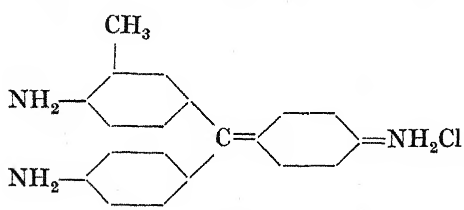
A "neutral" dye-salt may clearly also be formed by combination between a colour-base and colour-acid. These compounds are for the most part insoluble in water, although soluble in alcohol. Being of high molecular dimensions, they have the properties of colloids, amongst others, that of forming permanent colloidal solutions in the presence of excess of either component. Moreover, the composition of the dye that is precipitated varies according to the relative proportion of the two reagents in the solution.

These dyes are used especially in distinguishing between the various kinds of leucocytes in blood (see Ehrlich and Lazarus, *Die Anœmie*, Wien, 1898); but are coming of later years to be applied to various other purposes.

238. Influence of Side-chains. Almost any dye can be modified in its characteristics by the introduction of side-chains. Most commonly these are methyl or ethyl radicals, but they may be even more complex (*e.g.* additional benzene rings). The usual effect of an added side-chain is to deepen the shade or even to change the colour to one of deeper hue. The influence on colour differs according to where these side-chains are introduced in the dye molecule. Thus pararosanilin chloride is changed to rosanilin chloride



Pararosanilin



Rosanilin

by introducing a methyl group directly into one of the benzene rings. Rosanilin is slightly deeper in shade than pararosanilin. The shade may be further deepened by introduction of another methyl group into each of the other two rings, the resulting dye (new fuchsin) being deeper yet, but still red in hue. If, however, the methyl substitution is effected by replacing the H-atoms of the NH_2-groups, the hue is changed to violet, a reddish-violet if only two or three methyl radicals are introduced, but a blue-violet if all six amino H-atoms are thus replaced. Finally, it is

possible by converting one of the trivalent N atoms into the pentavalent form to introduce another methyl group, and methyl green results ; *i.e.* the colour has been changed to that of deeper hue, namely, green.

This principle applies in practically any group of dyes, and may often be taken into account to advantage in the search for some new stain to give a particular desired effect.

239. The Nature of the Staining Process. From what has been said in the preceding paragraphs it may be realised that a solution of a dye is a complex system from a physico-chemical standpoint. Moreover, the structures to be stained are present as separate phases, solid or liquid, of a heterogeneous system. Much discussion has taken place with respect to the process of dyeing, and various theories of its nature as being essentially chemical or essentially physical, in the sense of adsorption, mechanical or electrical, or in the sense of solid solution, involving partition between the solution and the tissue elements according to relative solubility of the dye therein, have been advocated. It is probable that all these factors play their part in varying proportion and that no one theory alone can explain all the facts.

We shall be in a better position to appreciate the complexity of the conditions present if we examine, to begin with, the case of a pure substance, cellulose, in relation to pure solutions of an acidic and a basic dye respectively.

We take, then, a piece of the purest analytical filter paper, wash it with distilled water to remove possible traces of acid, and place it in a dilute solution of the acid dye Congo red, freed from foreign salts. It is scarcely stained at all. Add next a very small amount of a neutral salt, say, sodium chloride. The paper is deeply stained. How are these facts to be explained ? In view of the chemical inertness of cellulose, it seems unlikely that a chemical combination occurs between the dye and the paper under the influence of a neutral salt at ordinary temperatures. Moreover, the same behaviour is shown by such different substances as charcoal, silk, alumina, silica, and so on. The process must be one of adsorption or deposition of the dye on the surface by some means. In other words, it must be associated with the decrease of surface energy of some kind. In the absence of foreign electrolytes, adsorption may be due to decrease of surface energy of the ordinary kind, known as surface tension. This is confirmed by the fact that the dye, in the absence of electrolytes, can be washed out again by water. But since the degree of staining is very small, there must be some influence at work restricting the mechanical adsorption. There is, indeed, another property of the boundary surfaces between phases which demands attention here. This is the electrical charge, nearly always present. If we test paper in water, we find that it has a negative charge. Similarly,

by appropriate means, we find that the dye itself has a negative charge. Hence there are repellent forces acting between the dye and the paper. Suppose, however, that we have also present the ions into which a neutral salt dissociates. The cations, being positively charged, are deposited on the surface of the paper, decreasing or annulling its negative charge and reducing the free energy. There is now little or no obstacle to the adsorption of the dye.

Turning now to the basic dyes, we find that the paper is the more deeply stained the *lower* the concentration of salt present. According to Freundlich, it is the colour base that is chiefly adsorbed in this case. As was pointed out above, these dyes are hydrolytically dissociated, so that free base is present. This free base, being insoluble, is in the colloidal state, and, like colloidal bases in general, has a positive charge, due to electrolytic dissociation of the surface of the particles. See HARDY in *Van Bemmelen Gedenkboek*, p. 188. Thus, not only are the coloured ions, in this case the cations, strongly adsorbed by the negative paper, but the free base is also. Since foreign electrolytes diminish the charge on the paper, their effect on staining by basic dyes is naturally of the opposite kind to that described in the case of the acidic dyes. The effect of alcohol is in the same direction as that of electrolytes, since it also decreases the electric charge and, therefore, the amount of dye adsorbed. Facts of the kind referred to in the preceding statements have given rise to an "Electrical Theory of Dyeing," which may account for a larger number of them than any other single theory is able to do. For further particulars of theories and facts relating to dyeing and staining, the reader is referred to ALFRED FISCHER's *Fixirung, Färbung und Bau des Protoplasmas*, Jena, 1899; PELET-JOLIVET's *Theorie des Farbeprozesses*, Dresden, 1910; *First Report on Colloid Chemistry*, Brit. Ass., 1917; GEE and HARRISON, *Trans. Faraday Soc.*, vol. vi, 1910; HARRISON, *Journ. Soc. Dyers and Colourists*, December, 1911; BAYLISS, *Biochem. Journ.*, vol. i, 1906, p. 175.

The above is by no means the only theory of staining. A recent discussion on the subject (HOLMES, *Stain Technology*, vol. iv, 1929, p. 75; STEARN and STEARN, *Stain Technology*, vol. v, 1930, p. 17) is of interest to those readers wishing to go deeper into the subject. Much of the discussion relates to the question whether to explain the phenomena on a chemical or physical basis. The problem is complicated by the impossibility of distinguishing completely between chemical and physical forces. Thus the electrical theory outlined above and explained on physical terms is not very different from the usual chemical theory, namely, that basic dyes have an affinity for the acid parts of the cell, acid dyes for the basic structures. The usual purely physical theory assumes that dyes are fixed to the tissue by non-electrical adsorp-

tion, much as charcoal adsorbs colour from solutions on which it is employed as a decoloriser. The physical theories of staining are well presented by Holmes in the paper cited above, and are very generally held by dye chemists at present. Chemical or electrical theories, on the other hand, are favoured by some biochemists for the reasons pointed out by the Stearns.

240. Removal of Dyes. One strong argument in favour of chemical or electrical fixation of dyes is the difficulty with which they are ordinarily removed. On the electrical theory above outlined one can explain this by assuming that the dye can only be set free by reversing the sign of the charge on the surface. This cannot be done by pure water alone. It can be done, however, by acid or alkali in the appropriate case. For example, if an acidic dye has been fixed on a negative surface by the aid of cations, which convert the charge to a positive one, OH' ions, provided by alkali, are powerful enough to change the sign of the charge back again to negative and thus free the dye, whereas $H^{\cdot}$ ions from an acid only increase the positive charge and fix the dye more firmly. Hence the statement that acidic dyes are fast to acids. A basic dye, adsorbed by a negative surface, is removed by acids and intensified by alkalies. A corresponding explanation holds. Thus, $H^{\cdot}$ ions from acids make the surface more positive, hence the dye is released. OH' ions make it more negative, hence the dye is held faster. In all cases, if the acid or alkali is strong enough, any dye-salt adsorbed is decomposed, sometimes with change of colour.

The process of "differentiation" by alcohol or other agent, to be referred to below, is an application of these facts. Alcohol removes a "basic" dye because it reduces the negative charge of the tissue elements and thus releases part of the positively charged constituent of the dye adsorbed.

241. "Specific" Stains. Certain tissue elements and cell-constituents have the property of staining deeply with particular dyes. That of nervous structures with methylen blue and of mitochondria with dyes containing di-ethyl-safranin, such as Janus green, may be given as examples. The property may be shown either by their taking up the stain from a dilute solution more rapidly than other structures present do ("progressive" staining), or by their holding on to it more tightly when excess of general stain is washed away by appropriate treatment. This latter process is sometimes known as "differentiation" or as "regressive" staining.

It is natural to interpret this behaviour as due to a chemical combination of a special kind, as did Ehrlich in his well-known theory of "chemo-receptors," according to which certain "side-chains" of protoplasmic molecules have special affinities for particular groups in the dye molecules. While this may be the

case in isolated instances, there are many facts which show that it cannot be accepted as a general law. It is difficult to see what purely chemical relationship can exist between complex, substituted, diazo-sulphonates, as a large number of these specific dyes are, and the chemical components of cells. Moreover, although methylen blue and other thiazines are specific vital stains for nervous tissue, certain safranin azo-dyes—diazin-green, for example—which have no chemical relationship to the former, are also vital nerve stains ; while similar compounds of the safranin series itself have no such property. See MICHAELIS, *Chemie der Farbstoffe*, 1902, p. 104. We have already seen how complex are the physical factors that intervene in such a simple case as the staining of paper, and to these may be added questions of solid solution, distribution between phases, diffusibility, and so forth. Indeed, it would seem that each individual case of specific staining requires investigation by itself.

242. Objects of Staining. Most constituents of cells are, in their natural state, either colourless or only faintly coloured. Thus they are only visible if their refractive indices differ from those of the media in which they are immersed. Such, for example, are fatty globules and the granules of many secreting cells. But, as seen thus, it is not an easy matter to judge of their true forms. This is greatly facilitated by staining them either more deeply than, or of a different colour from, their surroundings. If colourless glass beads, although they are easily seen by refraction, could only be observed from the direction of a line through the hole in the centre, the recognition of their true form would be difficult. Immersing them in a medium of the same refractive index as themselves would render them invisible. But if they were made of coloured glass and immersed in such a medium, they would be readily detected and their shape recognised.

The chief object of histological staining is then to cause certain constituents of the cells to take on a different intensity of tint from others. This may be done in various ways, as will be seen later. It is usual to distinguish two kinds of selective staining, *histological* and *cytological* selection. In the former an entire tissue or group of tissue elements is prominently stained, the elements of other kinds present remaining colourless or being differently stained, as in the impregnation of nerve endings by the silver and gold reduction methods. In the latter the stain is taken up or retained by some constituent element of the cell, such as the chromatin of the nucleus or an element of the cytoplasm.

The nuclear stains are of importance in marking out the contours and relations of the tissues making up regions or organs as a whole, and are thus of special value to the embryologist and morphologist.

At one time, it was thought to be possible to distinguish between "basophilous" and "acidophilous" tissue elements, according to their affinity for basic or acidic dyes. EHRLICH (*Du Bois Reymond's Archiv.*, 1879, p. 571) thought that the basic dyes have a special affinity for the chromatin of nuclei and the acidic dyes for the cytoplasm and intercellular substances. But we have already seen that the same substance may take up either kind of dye, according to the conditions present. Most staining processes are undertaken on cells which have been acted on by fixing reagents or by the so-called "mordants," and these may reverse the natural behaviour to dyes. EHRLICH's statement only applies in fact to cover-glass preparations fixed and dried by heat, without the action of reagents. The acidic colours, orange and acid fuschin, although they stain cytoplasm, may give good chromatin differentiation when used as regressive stains. Methylen blue is basic, but stains nerves. It has been shown in fact, that by pre-treatment with solutions buffered to various reactions, tissue normally acidophilic may be stained with a basic dye, and one normally basophilic with an acid dye.

243. Intra-vitam Staining. It is clear that unless the cell-membrane of a living cell is permeable to a dye, no constituent of the cell can be stained by it. Most dyes appear to be more or less toxic if they enter the cell. But, while alive, the latter is to a large extent protected, since the dye may not obtain entrance. A living *Amœba* is stained by very few dyes. Neutral red, however, passes through the membrane and stains various structures, while having no apparent effect on the activities of the organism. The auricle of the frog's heart can also be stained with this dye, while continuing its normal contractions. Used in this way, the dye is applied in very dilute solution. Since neutral red is a very sensitive indicator just about the neutral point, the fact of its permeability and non-toxicity makes it a valuable test for the presence of acid or alkali within the cell. But neutral red in very dilute solution will inhibit nutosis as shown by v. MÖLLENDORF.

When a dye enters a living cell, it usually stains various granules and structures contained therein, while at the same time it is uniformly diffused through the liquid phase of the protoplasm. If the process of staining is conditioned by phenomena at boundary surfaces, simple undifferentiated protoplasm in the living state should be incapable of staining, and this seems to be the general experience. As regards the question of permeability to a given dye, unless the cell is able to show that it is still alive by movement or by contractility, it is clearly a matter of difficulty to be certain that, when a particular dye enters, it does so during life or only after it has destroyed the normal properties of the cell-membrane.

The nucleus itself seems to be very resistant to dyes while alive, and it has been stated that the appearance of stain in it is a sure indication of death. BOLLES LEE made a large number of observations and came to the conclusion that most of the " intra-vitam " stains are either due to mere diffusion through the liquid protoplasm or that the stained constituents were not really living, being food particles or products of cell activity.

At the same time, many of the methods which come under this heading are of much value. Methylen blue may be injected into the living animal and frequently gives very successful staining of nervous structures, owing to the fact of its being conveyed into intimate contact with the cells by means of the blood vessels.

The various methods of preserving the stain in the structures to which it was localised during life obviously depend on the adequacy of the means used to fix and maintain these structures and to retain the properties owing to which the stain was taken up. This is by no means a simple matter. MOTT describes in living nerve cells a number of minute particles which stain on the outside with methylen blue. In fixed cells, as is well known, these particles aggregate together to form the " Nissl granules." MICHAELIS found similar granules in liver cells. As the cells die, the stain leaves the granules and passes into the nucleus.

The behaviour of the living nucleus to methyl green has given rise to some discussion. It appears that no uni-cellular organism in which the nucleus was so stained has been observed to move, whereas the chlorophyll grains may take up the stain while the cell is normally motile.

The question as to whether the cell elements which stain during life are to be described as living or not is scarcely putting the problem from the right point of view. If a dye obtains contact with the interfaces between constituents of a cell, it will in all probability be deposited there to a degree depending on the various properties of the interface described previously. This may occur independently of the fact as to whether one or both of the phases is living.

The details of the various methods used for intra-vitam staining and the fixation of the results are described in other parts of this book. The reader may be referred to the work of GOLDMAN (*Unters. ueber die Sekretion des Organismus im Lichte der " vitalen Färbung,"* Laupp ; Tübingen, 1912) for certain aspects of the problem. A splendid summary is given by CAPPELL, (*J. Path. and Bact.*, vol. 32, 1929).

244. Fixed Tissues. The majority of staining methods are undertaken on tissues that have been fixed and hardened by reagents. It is sufficient to mention here that some of these reagents merely serve to coagulate or precipitate the constituents of cells without marked changes in their chemical nature, although

their physical state is more or less altered. Alcohol is one of these agents. Other fixing fluids, of which those containing chromic acid are representatives, produce what seem to be compounds of cell proteins with the reagent. In this latter case, we have what is known as a " mordant " present.

Mordants are usually understood to be agents which form insoluble compounds with dyes and in this way cause their fixation in places from which otherwise they might be washed out by the subsequent treatment with dehydrating agents, etc. Such a substance may clearly be either already present in the fixed preparation when the dye is added, or it may be added together with or subsequently to the staining agent. It will readily be understood that the production of a hardly soluble dye salt renders solution in water more difficult, but this alone is not enough. The compound must also be firmly attached to the surface.

The insoluble compounds between a dye and a mordant are known as " lakes." But the chemical nature of these substances is by no means clear—especially when they are produced *in situ* in stained tissues. In fact, they do not behave as simple compounds of the dye and the mordant. They resist the action of strong acids and bases in moderately strong solutions, so that the stains obtained by this method are characterised by durability and " fastness."

On the whole, we must conclude that there are factors still unknown, but possibly related to the electrical properties of the surface, which play an important part in these reactions with mordants. Neither a simple chemical theory nor one purely physical accounts for all the phenomena.

A further mention may here be made of the " progressive " and " regressive " methods. A preparation may be placed in a very dilute solution of a dye and the action stopped when the elements with the greatest " affinity " for the dye have taken it up. The staining of nuclei with dilute alum-hæmatoxylin may be mentioned. If the action is prolonged, various other constituents of the cell, besides the nucleus, take on the colour. As a rule, no great differentiation is obtained by this method. Better results are given by the " regressive " method, in which a general overstaining is followed by a partial decoloration, in which certain elements retain the stain, owing to special chemical or physical properties, after it has been removed from the rest. Thus safranin stains the whole section a deep red colour; but alcohol removes the stain from all but the chromatin and the nucleoli. This action of alcohol may be explained, as already pointed out, by its effect on the magnitude of the electric charge, since its dielectric constant is lower than that of water. Other differentiating agents are also used. Iron-alum, in the

iron-hæmatoxylin method, serves both as preliminary mordant and as differentiating agent. This double action is not easy to explain and confirms what was said above as to the complexity of the process.

245. Metachromasy. There are a few dyes, mostly of the basic anilin series, which stain certain elements in the colour of the ordinary solution of the dye, other elements in that of the free colour-base. Safranin stains nuclei red; mucin and the ground substance of cartilage, orange. Methyl violet stains "amyloid," and mucin red.

Although in a few cases this behaviour may be due to the dye being really a mixture of two dyes, as in the case of iodine green, there is no doubt that this is not the explanation of genuine cases. According to MICHAELIS, the appearance of the colour of the base is not due to the alkalinity of the elements in question. The fact that the red stain given to mucin by thionin can be changed into blue by alcohol and black to red by water shows that the change is not one involving great alternations of chemical structure, and a tautomeric one is naturally suggested. It would appear that the change is one by which an amino-group becomes freed from its combination with the mineral acid of the salt. HOLMES (*Stain Technology*, vol. i, 1926) has shown that in such a case an addition product may result in which the pentavalent nitrogen becomes trivalent, with corresponding change in colour. In the case of thionin, the acid is supposed to change its connection to the nitrogen which unites the two benzene rings. What conditions regulate the change from one form to the other are unknown. A similar difficulty is met with in the case of iodine, which is brown in solution in alcohol, violet in chloroform.

We must, however, not overlook the changes in colour shown by substances in the colloidal state merely in consequence of a decrease in their degree of dispersion or increase in size of particles. Gold is a notable case. It may be red, violet, blue or green. All of these tints are met with in its use as a histological reagent. Whether similar phenomena may occur in the adsorption of dyes is uncertain, but cannot be dismissed without further evidence.

246. The Use of Stains in Practice. Stains for special purposes are described in other pages of this book. It will be obvious from the contents of the present chapter that caution must be exercised in making deductions as to chemical composition from behaviour to dyes.

The most distinctive processes, involving the use of mordants and regressive differentiation, can only be undertaken on sections. Staining in bulk is useful when the general anatomy is the object of study. For cytological work it is of little value.

247. *As remarked, the main object of staining is to demonstrate the structures present in the cell. The fact, however, that this appearance is not necessarily that of the living state should never be allowed to escape remembrance. Without special investigation of the case, it is not permissible to draw conclusions as to the chemical nature of a cell constituent from its behaviour to dyes.*

CHAPTER XIV

CARMINE AND COCHINEAL STAINS

248. Carmine. This dye is obtained from the ground-up bodies of the cochineal insect and since the latter varies greatly in the quality of the product yielded, and different methods are used to extract it, the powdered carmine obtained in commerce is extremely variable. For ordinary histological staining this variability may be of little importance, but for techniques requiring a high degree of specificity, such as aceto-carmine, the use of a first-class product is essential for satisfactory results.

Carmine is by no means merely carminic acid with at most certain impurities. According to the analysis of LIEBERMANN (*Ber. d. Chem. Ges.*, Jahrg. 18, 1886, pp. 1969—1975) it is a *very peculiar alumina-protein compound of carminic acid*, a true chemical compound from which at all events *aluminium* and *calcium* can no more be absent than sodium from salt. It results from the researches of MAYER (*Mitth. Zool. Stat. Neapel,* x, 1892, p. 480) that in the processes of histological staining (*not* of industrial dyeing) the active factors of the compound are, besides the carminic acid, always the *alumina*, and in some cases the lime. *The other bases are inactive;* the nitrogenous matters, so far as they have any influence at all, are an obstacle, as it is they that give rise to the well-known putrefaction of the solutions.

This being so, it follows that *carminic acid* may, if desired, *be taken as the basis of staining solutions instead of carmine*. Staining solutions thus prepared do not give essentially better stains than those made with carmine; but have the advantage of being of more constant composition.

Carminic acid is soluble in water and *weak* alcohol (that of 70 per cent. only dissolves less than 3 per cent.). It cannot be used alone for staining, as it only gives in this way a weak and diffuse stain.

249. Cochineal. According to MAYER (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 496), the active principle of extract or tincture of cochineal (as used in histology) is not free from carminic acid chemically combined with a base which is not lime, but some alkali. The watery extract made with *alum*, or cochineal alum-carmine (§ 275) owes its staining power to the formation of carminate of alumina (last §). The tincture made with *pure alcohol,* on the other hand, contains only the above-mentioned carminate of some alkali. This carminate *alone* stains weakly and diffusely

(like carminic acid alone). But if in the tissues treated with it, it meets with lime salts, alumina or magnesia salts, or even metallic salts capable of combining with it and forming insoluble coloured precipitates in the tissues, then a strong and selective stain may result. And if the necessary salts be added to the tincture itself there results a solution containing the necessary elements for affording a strong and selective stain with all classes of objects Hence Mayer's later formula, § 276.

250. General Remarks. Carmine stains are widely used, at the present time, for three general purposes. Because of the great permanence of this dye in balsam, embryologists use it for staining sections of valuable embryos. As a stain for whole objects, carmine is about the most satisfactory dye because it does not overstain very readily and the excess is easily removed. Aceto-carmine is very widely employed by cytologists for studying, in fresh tissue, the chromosomes of both animals and plants.

Grenacher's alcoholic borax-carmine may be recommended to the beginner as being the easiest of these stains to work with: or para-carmine, for objects which require a strong alcoholic solution. Carmalum, or one of the alum-carmines, is also an easy and safe reagent.

Overstains may in all cases be washed out with weak HCl (*e.g.* 0·1 per cent.). Alum-solution will often suffice, or, according to Henneguy (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 400), permanganate of potash. The alum-carmines are fairly permanent in glycerin. None of the acid stains, nor any of Grenacher's fluids, should be used with calcareous structures that it is wished to preserve, unless they be taken in a state of extreme dilution.

A. AQUEOUS CARMINE STAINS

a. Acid

251. Alum-carmine (Grenacher, *Arch. mik. Anat.*, xvi, 1879, p. 465). An aqueous solution (of 1 to 5 per cent. strength) of common or ammonia alum is boiled for ten to twenty minutes with $\frac{1}{2}$ to 1 per cent. of powdered carmine. (It is perhaps the safer plan to take the alum solution highly concentrated in the first instance, and after boiling the carmine in it dilute to the desired strength.) When cold, filter.

Alum-carmine is an *excellent* stain. It is particularly to be recommended to the beginner, as it is easy to work with; it is hardly possible to overstain with it. Its chief defect is that it is *not very penetrating*, and therefore unsuitable for staining objects of considerable size in bulk.

This stain must be avoided in the case of calcareous structures that it is wished to preserve.

Tizzoni (*Bull. Sc. Med. Bologna*, 1884, p. 259), Pisenti (*Gazz. degli Ospetali*, No. 24; *Zeit. wiss. Mik.*, ii, 1885, p. 378) and Grieb (*Mem. Soc. Ital. Sci.*, t. vi, No. 9, 1887; *Zeit. wiss. Mik.*, vii, 1, 1890, p. 47), have given modifications of Grenacher's formula which do not appear to us rational.

Mayer (*ibid.*, xiv, 1897, p. 29) makes a stronger stain by taking 2 grm. carmine, 5 grm. alum, and 100 c.c. water, and boiling for an hour.

252. **Acetic Acid Alum-Carmine** (Henneguy, in *Traité des Méth. Techn.*, Lee et Henneguy, 1887, p. 88). Excess of carmine is boiled in saturated solution of potash alum. After cooling add 10 per cent. of glacial acetic acid, and leave to settle for some days, then filter.

For staining, enough of the solution is added to distilled water to give it a deep rose tint. In order to ensure rapid diffusion it is well to bring the tissues into the stain direct from 90 per cent. alcohol. Stain for twenty-four to forty-eight hours, and wash for an hour or two in *distilled* water. Mount in balsam. You can mount in glycerin, but the preparations do not keep so well.

The advantage of this carmine is that it has much *greater power of penetration* than the non-acidified alum-carmine.

253. **Cochineal Alum-Carmine** (Partsch, *Arch. mik. Anat.*, xiv, 1877, p. 180). Powdered cochineal is boiled for some time in a 5 per cent. solution of alum, the decoction filtered, and a little salicylic acid added to preserve it from mould.

Another method of preparation has been given by Czokor (*ibid.*, xviii, 1880, p. 413). Mayer finds that Partsch's is the more rational, the proportion of alum in it being exactly right, whilst in Czokor's it is insufficient. Partsch's fluid also keeps better.

Rabl (*Zeit. wiss. Mik.*, xi, 2, 1894, p. 168) takes 25 grm. each of cochineal and alum, 800 c.c. of water, and boils down to 600 c.c. He prefers this because it is *not so purely nuclear* a stain as the others.

These solutions give a stain that is practically identical with that of alum-carmine made from carmine, with perhaps even more delicate differentiations.

Rawitz (*Zeit. wiss. Mik.*, xxv, 1909, p. 392) takes cochineal 4 grm., nitrate of aluminium (or ammonio-sulphate of cobalt) 4 grm., water 100 c.c. and glycerin 100 c.c. Only for sections.

254. Mayer's **Carmalum** (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 489). Carminic acid, 1 grm.; alum, 10 grm.; distilled water, 200 c.c. Dissolve with heat (if necessary). Decant or filter. Add some antiseptic, either 1 c.c. formol, or 0·1 per cent. salicylic acid, or 0·5 per cent. salicylate of soda. The solution will then keep. It *stains well in bulk*, even osmium objects. If washed out with distilled water only, the plasma will remain somewhat stained. If this be not desired, wash out carefully with alum solution, or, in difficult cases with weak acid, followed in either case with water. The general effect is that of an alum-carmine stain.

A weaker solution may be made by taking from three to five times as much alum and five times as much water, and dissolving in the cold.

With either solution the objects to be stained should *not* have an *alkaline reaction.*

Rawitz's Carmalum (*Anat. Anz.*, xv, 1899, p. 438). Ammonium alum, 20 grm.; distilled water, 150 c.c.; glycerine, 150 c.c.; carminic acid, 2 grm. The ammonium alum should first be dissolved in the distilled water, then the carminic acid added,

and the mixture heated to assist in dissolving. After cooling the glycerine is added, and the mixture filtered. Recommended by Ludford for counterstaining trypan blue material (see § 750). Keeps well, only for sections.

All solutions prepared with alum tend to precipitate. Carmalum made up with 500 c.c. of water instead of 200, and with glycerin or 10 per cent. of formol or pyroligneous acid added, keeps well.

255. Mayer's **Aqueous Aluminium-Chloride Solution** (*Mitth. Zool. Stat. Neapel*, x, 1902, p. 490). Carminic acid, 1 grm.; chloride of aluminium, 3 grm.; water, 200 c.c. Add an antiseptic, as for carmalum.

Use as carmalum. The stain is of a blue-violet colour, very powerful and elective, but not so purely nuclear as carmalum. It is recommended only as a substitute for carmalum in cases in which the latter is counter-indicated on account of the alum in it or the like.

256. Alum-Carmine and Picric Acid. Alum-carmine objects may be double-stained with picric acid. Legal (*Morph. Jahrb.*, viii, p. 353) combines the two stains by mixing 10 vols. of alum-carmine with 1 of saturated picric acid solution. I find this very recommendable.

257. Aceto-Carmine (Acetic Acid Carmine) Schneider (*Zool. Anz.*, 1880, p. 254). To boiling acetic acid of 45 per cent. strength add carmine until no more will dissolve. Cool and filter. With some batches of carmine it is much better to simmer an excess of carmine in 45 per cent. acetic acid, preferably under a reflux condenser, for an hour or two, and after cooling filter. (According to Schneider, the largest proportion of carmine is dissolved in acetic acid of 45 per cent. strength. Usually less than ½ grm. of carmine will dissolve in 100 c.c. of 45 per cent. acetic acid.)

Belling's Iron Aceto-Carmine. Belling found that the addition of a trace of iron to Schneider's aceto-carmine makes the chromosomes, in fresh tissue, stain more deeply (*Amer. Nat.*, vol. 55, 1921, p. 573). If the tissue requires teasing in order to separate the elements, this is done on a glass slide in a few drops of aceto-carmine with steel needles and usually enough iron will be dissolved to serve as a mordant. Or a few drops of ferric hydrate, dissolved in 50 per cent. acetic acid, is added to ordinary aceto-carmine until it becomes a bluish-red, but without a visible precipitate. Then add an equal part of untreated stain.

The amount of iron needed to give the optimum effect varies with the tissue used and the sample of carmine employed to make aceto-carmine in the first place Too much iron will completely spoil the stain so it is well to go slowly, adding minute traces of iron each time until a satisfactory stain is obtained for the particular material in hand.

Aceto-carmine, either with or without iron, may be used in a 1 per cent. strength as a slow stain, but, for studying chromosomes in fresh tissue, full strength is required. The procedure varies in details but, in general, the method of application is as follows: Fresh material is placed on a glass slide and aceto-carmine is

added. If teasing is required, this is now done. A thin coverslip is then placed over the material which is allowed to stain from a few minutes up to half an hour or more, depending on the sample of stain and the tissue. The excess stain is drawn off with filter paper and the tissue is crushed so that the cells will not be more than a few layers thick. All excess stain is now blotted off, and the edge of the coverslip may be sealed with vaseline, melted paraffin or even thick damar. If only a temporary mount is wanted the sealing can be omitted, since a well-made aceto-carmine slide will keep well for a few hours without sealing.

Aceto-carmine has proved to be an exceedingly valuable cytological reagent for a study of chromosomes in fresh tissues. It fixes and stains the chromosomes at the same time, so that they may be studied at once without recourse to the ordinary methods of preservation and sectioning. For making chromosome counts and for a study of the morphology of the metaphase chromosomes either in mitosis or meiosis, it is excellent. Recently, several different methods have been devised for making permanent mounts of aceto-carmine preparations. While in the writer's experience these are not quite as brilliant as fresh slides, they are entirely satisfactory for all but the most detailed type of observation.

Too much emphasis cannot be placed upon the fact that the successful use of aceto-carmine depends upon having the proper grade of powdered carmine to begin with. Unfortunately, there is no method by which we can test the carmine except its use. If the reader experiences unsatisfactory results he is advised to try other samples of carmine.

The study of aceto-carmine mounts is facilitated if the observer will use a blue-green filter for artificial light. For the 6-volt research lamp in common use in America, the Zeiss B-G No. 7 filter will be found excellent when used with a frosted glass screen.

Methods for Making Permanent Aceto-Carmine Slides

McClintock (*Stain Technology*, iv, 1929, p. 53) first preserves plant material in acetic-alcohol. The contents of anthers are squeezed out on a slide in a few drops of Belling's aceto-carmine and covered. The slide is heated over an alcohol lamp for a second, repeating four or five times. Now place the slide in a Petri dish containing 10 per cent. acetic acid, until the coverglass will come free. Then pass the slide and the coverglass separately through the following solutions : 1 part glacial acetic acid and 3 parts absolute alcohol; then 1 part of acetic and 9 of absolute alcohol; and then absolute alcohol. From this it is passed through xylol and the slide and cover are reunited in damar or balsam.

Steere (*Stain Technology*, vi, 1931, p. 107) makes smears of fresh anthers, stains these in steaming iron aceto-carmine for

from one to ten minutes. The slides are now passed rapidly through the following solutions: 1 part of glacial acetic acid and 2 of absolute alcohol; then 1 part of acetic to 9 of absolute alcohol. Dehydration is completed in pure absolute alcohol and the slide cleared in xylol and mounted.

For animal tissue the same method which McClintock follows may be used. The coverglass is floated off fresh aceto-carmine preparations in 10 per cent. acetic acid and carried through the steps of dehydration and mounting she uses for plant tissue.

Buck (*Science*, lxxxi, 1935, p. 75) inverts an aceto-carmine slide (with supports) in a Petri dish containing equal parts of glacial acetic acid, absolute alcohol and xylol, until the coverglass soaks off. Then the slide and coverglass are passed through two changes of equal parts of absolute alcohol and xylol and then placed in pure xylol, from which they may be reunited in damar or balsam. Buck points out that Metz and Gay found that clove oil could be substituted for xylol in the initial steps.

258. **Lee's Iron Carmine.** We recommend trial of the following, which Lee has already published in the *Traité des Meth. Techniques*, Lee et Henneguy, 1902. Sections are mordanted (a few hours will suffice) in sulphate of iron (Benda's *liquor ferri*, as for iron hæmatoxylin), washed, and stained for an hour or so in 0·5 per cent. solution of carminic acid in alcohol of 50 per cent. Wash in alcohol of 50 per cent.; no differentiation is necessary. When successful, there results an almost pure chromatin stain, quite as sharp as iron hæmatoxylin, but somewhat weak.

Iron Carmine. Pfeiffer von Wellheim (*Zeit. wiss. Mik.*, xv, 1898, p. 123) mordants for six to twelve hours in a very weak solution of chloride of iron in 50 per cent. alcohol, washes in 50 per cent. alcohol, and stains as above. Overstains may be corrected with 0·1 to 0·5 per cent. HCl alcohol. Lee found this good, but not so good as the last.

Iron Carmine (Zacharias, *Zool. Anz.*, 1894, p. 62). Stain for several hours in an aceto-carmine (made by boiling 1 grm. of carmine with 150 to 200 c.c. of acetic acid of 30 per cent., for twenty minutes, and filtering). Rinse the objects with dilute acetic acid, and bring them (taking care not to touch them with metallic instruments) into a 1 per cent. solution of ammoniated citrate of iron. Leave them, for as much as two or three hours if need be, till thoroughly penetrated and blackened (with sections this happens in a few minutes). Wash for several hours in distilled water. A chromatin and plasma stain.

259. **Hollande's Chlorcarmine Staining Method** (*C. R. Soc. Biol.*, 1916, lxxix, p. 662, and *Jour. Roy. Micr. Soc.*, 1920). Place 5 c.c. pure hydrochloric acid in a porcelain dish; add little by little 14 grm. powdered carmine, stirring constantly to make a homogeneous doughy mass. Allow to digest for twenty-four hours; add 250 c.c. aq. dest., bring to the boil, and keep boiling for half an hour. Filter; make up to 180 c.c. with aq. dest., and then add enough 75 per cent. alcohol to make a total volume of 200 c.c. Stain sections or pieces of tissue for two to twenty-

four hours. Rinse in aq. dest. or 30 per cent. alcohol; immerse in 3 per cent. iron alum solution, in which the sections become black, and are then slowly decolorised; when differentiation is complete, rinse in a 1 per cent. pyridin solution, and wash under the tap for ten to fifteen minutes. Counterstain and mount as desired. This is a very intense stain suitable for mitochondria and cell granules. See also § 229.

260. Iron Carmalum (DE GROOT, *Zeit. wiss. Mik.*, xx, 1903, p. 21). Dissolve 0·1 grm. of ferric alum in 20 c.c. distilled water and add 1 grm. carminic acid. Dissolve, add 180 c.c. of water, warm, add 5 grm. potash alum, dissolve, cool, filter, and add 2 drops of hydrochloric acid. To be used as carmalum, and said to give a stronger stain.

261. Iron Cochineal (SPULER, *Encyclopædie d. mik. Technik*, 1903, p. 153, and 1910, p. 240). Stain for forty-eight hours in an incubator in extract of cochineal (made in a highly complicated way), wash with water, put into solution of ferric alum of $\frac{3}{4}$ per cent. strength for twenty-four hours or more. If the stain is not sufficiently intense, the whole process may be repeated.

PETER (*Zeit. wiss. Mik.*, xxi, 1904, p. 314) stains material in bulk for forty-eight hours (sections eighteen to twenty-four) in an incubator, in a similar extract, acidified with HCl, treats with iron-alum of $2\frac{1}{2}$ per cent. for one hour to one day (sections half to two minutes), then alcohol, xylol, paraffin, or balsam. Chromatin black, protoplasm grey, *yolk granules red.*

HANSEN (*ibid.*, xxii, 1905, p. 85) stains sections or entire objects in a solution of 5 to 10 grm. cochineal, 8 grm. ferric alum, 250 c.c. water, and 25 c.c. sulphuric acid of 10 per cent., boiled for fifteen to twenty minutes.

β. So-called "Neutral" and Alkaline

262. Ammonia-Carmine. Best made by the method of RANVIER. Make a simple solution of carmine in water with a *slight* excess of ammonia, and expose it to the air in a deep crystallising dish until it is entirely dried up. It should be allowed to putrefy if possible. Dissolve the dry deposit in pure water, and filter.

VAN WIJHE (*Vers. Akad.*, Amsterdam, viii, Deel, p. 507) takes an old strong solution of carmine in ammonia (or boils carmine with ammonia and peroxide of hydrogen), then precipitates it by adding alcohol to excess, washes the precipitate with alcohol, and dries it.

263. Soda-Carmine appears to be still used by some for central nervous system (see CUCCATI, *Zeit. wiss. Mik.*, iv, 1887, p. 50). It can be obtained from GRUBLER & HOLLBORN (*Natron-Carmin*).

264. ORTH'S Lithium-Carmine (see *early editions*) macerates strongly, and is superfluous. For that of BEST, see *Zeit. wiss. Mik.*, xxiii, 1906, p. 322.

265. Magnesia-Carmine (MAYER, *Zeit. wiss. Mik.*, xiv, 1897, p. 23). Take 1 grm. carmine, 0·1 grm. magnesia usta (freshly burnt), and 50 c.c. distilled water, boil for five minutes, filter, and add 3 drops of formol. This is the *stock* solution. A *weak* solution may be made by boiling 0·1 grm. carmine for half an hour in 50 c.c. of magnesia water (made by leaving 0·1 grm. of magnesia usta in contact with 100 c.c. of spring water for a week with frequent agitation, and decanting when required for use). Said to be less injurious to tissues than the other alkaline carmines.

266. As to Picro-Carmine. The term "picro-carmine" is commonly used to denote a variety of solutions in which carmine, ammonia, and picric acid exist *uncombined* in haphazard proportions. These solutions do *not* contain a double salt of picric and carminic acid and ammonia, or *picro-carminate of ammonia*. They are always alkaline, and frequently injurious to tissues. The *raison d'être* of picro-carmine does not lie in its capacity of affording a double stain, but in that the picric acid in it is supposed to neutralise the ammonia, which it only does imperfectly. See MAYER in *Zeit. wiss. Mik.*, xiv, 1897, p. 18.

267. RANVIER'S **Picro-Carmine, Original Formula** (*Traité*, p. 100). To a saturated solution of picric acid add carmine (dissolved in ammonia) to saturation. Evaporate down to one-fifth the original volume in a drying oven, and separate by filtration the precipitate that forms in the liquid when cool. Evaporate the mother liquid to dryness, and you will obtain the picro-carmine in the form of a crystalline powder of the colour of red ochre. It ought to dissolve completely in distilled water; a 1 per cent. solution is best for use.

For slow staining, dilute solutions may advantageously have 1 or 2 per cent. of chloral hydrate added to them.

Overstains may be washed out with hydrochloric acid, say 0·5 per cent. in water, alcohol, or glycerin.

Preparations should be mounted in balsam, or if in glycerin, this should be acidulated with 1 per cent. of acetic acid, or better, formic acid.

RANVIER'S **Later Formula** does not give a more constant product (see *previous editions*).

268. VAN WIJHE dissolves 0·5 per cent. of the dry ammonia-carmine, § 262, in a 1 per cent. solution of neutral picrate of ammonia, boils until the vapour ceases to blue reddened litmus paper, and adds 1 per cent. of chloral hydrate. Gives an almost neutral preparation.

269. MAYER'S **Picro-Magnesia Carmine** (*Zeit. wiss. Mik.*, xiv, 1897, p. 25) is relatively constant and innocuous to tissues. It consists of 1 vol. of the *stock* solution of magnesia-carmine (§ 265), and 10 vols. of a 0·6 per cent. solution of picrate of magnesia, or of equal parts of the *weak* solution and the picrate solution. The picrate may be obtained from GRUBLER & HOLLBORN, or the solution may be made by heating 0·25 grm. of carbonate of magnesia in 200 c.c. of 0·5 per cent. solution of picric acid, allowing to settle, and filtering.

DE GROOT'S picro-magnesia carmine (*ibid.*, xxix, 1912, p. 184) contains ammonia, which is bad, and seems to us superfluous.

270. **Other Formulæ for Picro-Carmine and Other Aqueous Carmines (Acid and Alkaline).** Lee has tried most of them, and found no real advantage in any of them (*see previous editions*).

B. ALCOHOLIC CARMINE STAINS

271. Alcoholic Borax-Carmine (GRENACHER, *Arch. mik. Anat.*, xvi, 1879, pp. 466 *et seq.*). Make a *concentrated* solution of carmine in borax solution (2 to 3 per cent. carmine to 4 per cent. borax) by boiling for half an hour or more (or allowing it to stand, with occasional stirring, for two or three days); dilute it with about an

equal volume of 70 per cent. alcohol, allow it to stand some time and filter.

Preparations should remain in the stain until they are thoroughly penetrated (for days if necessary), and then be brought (*without first washing out*) into alcohol *of* 70 *per cent.* acidulated with 4 to 6 drops of hydrochloric acid to each 100 c.c. of alcohol. They are left in this until they have taken on a bright transparent look (which may require days), and may then be washed or hardened in neutral alcohol. Four drops of HCl is generally enough. Three drops we find not quite sufficient.

For delicate objects, and for very impermeable objects, it may be well to increase the proportion of alcohol in the stain; it may conveniently be raised to about 50 per cent. It should not exceed 60 per cent. in any case (Mayer).

This stain used to be the most popular of any for staining in bulk. It is easy to use, and gives a most splendid coloration. But it is not so penetrating as is commonly supposed, and has the defect of sometimes forming precipitates in the cavities of bulky objects which cannot be removed by washing out. And the fluid is alkaline, and therefore may not be suitable for certain delicate work.

272. Lynch's Precipitated Borax-Carmine Method. According to Lynch (*Zeit. wiss. Mik.*, xlvi, 1929, p. 465) a much more selective and brilliant stain is obtained if, after staining overnight in Grenacher's borax-carmine, tissue is treated in the following way: Add cautiously to the dish containing the tissue and the dye, drop by drop, concentrated hydrochloric acid until all the carmine is precipitated out as a brick-red flocculent mass. Allow the dish to stand six to eight hours or overnight. The tissue is now placed in a 3 per cent. solution of HCl in 70 per cent. alcohol and destained until the cytoplasm is clear and the nuclei are pink in colour. This usually requires two or three hours. Wash out the acid with several changes of 80 per cent. alcohol, then dehydrate, clear and mount in the usual way. This method is suitable for objects which are to be mounted *in toto*, but it should not be used on delicate vesicular organisms as it will leave precipitates in their cavities.

273. Mayer's **Paracarmine** (*Mitth. Zool. Stat. Neapel*, x, 3, 1892, p. 491). Carminic acid, 1 grm.; chloride of aluminium, 0·5 grm.; chloride of calcium, 4 grm.; 70 per cent. alcohol, 100 c.c. Dissolve cold or warm, allow to settle, and filter.

Objects to be stained *should not have an alkaline reaction*, nor contain any considerable amount of carbonate of lime (spicules or skeletal parts of corals, etc.) which would give rise to precipitates. Wash out sections or objects intended to be sectioned, with pure 70 per cent. alcohol. Objects intended to be mounted whole may be washed out with a weak solution of aluminium chloride in alcohol, or if this be not sufficient, with 5 per cent. common acetic acid (or 2·5 per cent. glacial acetic acid) in alcohol.

This may also be done with section material, if it is desired to obtain a more purely nuclear stain.

For staining bulky objects with large cavities, such as *Salpa*, the solution should be diluted (with alcohol); and as this may cause precipitates to form during the staining, especially if the objects are not very clean, it is advisable to *slightly acidify the dilute solutions.*

Instead of calcium chloride, which is very hygroscopic, strontium chloride may be taken.

Paracarmine is less hurtful to delicate tissues than borax carmine; it is more highly alcoholic, therefore more penetrating; and has less tendency to form precipitates in the interior of objects. But, in our hands, it does not give quite so fine a stain.

274. **Alcoholic Hydrochloric-Acid Carmine.** GRENACHER'S receipt (*Arch. f. Mik. Anat.*, xvi, 1879, p. 468) is troublesome. That of MAYER (*Mitth. Zool. Stat. Neapel*, iv, 1883, p. 521; *Intern. Monatsschr. f. Anat., etc.*, 1897, p. 43) is better: Carmine 4 grm.; water, 15 c.c.; hydrochloric acid, 30 drops. Boil till the carmine is dissolved, add 95 c.c. of 85 per cent. alcohol, and neutralise by adding ammonia until the carmine begins to precipitate.

If it be desired to dilute the solution, it should be done with alcohol, not water, and alcohol of 80 to 90 per cent. should be taken for washing out.

A very *powerful* stain, which Lee has found useful. If it be desired to have a purely nuclear stain, the alcohol must be *very slightly* acidulated with HCl.

For a complicated receipt of LOEWENTHAL see *Zeit. wiss. Mik.*, xix, 1902, p. 56.

275. **Alcoholic Cochineal,** MAYER'S **Old Formula** (*Mitth. Zool. Stat. Neapel*, ii, 1881, p. 14). Cochineal in coarse powder is macerated for several days in alcohol of 70 per cent. For each gramme of the cochineal there is required 8 to 10 c.c. of the alcohol. Stir frequently. Filter.

The objects to be stained must previously be saturated with alcohol of 70 per cent., and alcohol of the same strength must be used for washing out or for diluting the staining solution. The washing out must be repeated with fresh alcohol until the latter takes up no more colour. Warm alcohol acts more rapidly than cold. Overstaining seldom happens; it may be corrected by means of 70 per cent. alcohol, containing $\frac{1}{10}$ per cent. hydrochloric or 1 per cent. acetic acid.

Small objects and thin sections may be stained in a few minutes; larger animals require hours or days.

This is a nuclear stain, slightly tinting the protoplasm. The colour varies with the reaction of the tissues, and the presence or absence of *certain salts* in them. Crustacea with thick chitinous integuments are generally stained red, most other organisms blue. The stain is also often of different colours in different tissue

elements of the same preparation. Glands or their secretion often stain grey-green.

Acids lighten the stain and make it yellowish-red. Caustic alkalies turn it to a deep purple.

All acids must be carefully washed out from the objects before staining, or a diffuse stain will result. The stain is permanent in oil of cloves and balsam.

Very penetrating and especially useful for Arthropoda.

It has over the later fluid (next §) the advantage of being more highly alcoholic; and it does not contain free acid, so that it *can be used with calcareous structures* which it is wished to preserve—which the later fluid cannot. For specimens of *Pluteus*, for instance, Lee found it excellent. But it only gives *good* results with such objects as contain the necessary salts, § 248.

276. Mayer's Alcoholic Cochineal, Later Formula (*Mitth. Zool. Stat. Neapel*, x, 1892, p. 498). Cochineal, 5 grm.; chloride of calcium, 5 grm.; chloride of aluminium, 0·5 grm.; nitric acid of 1·20 sp. gr., 8 drops; 50 per cent. alcohol, 100 c.c. Powder the cochineal and rub up with the salts, add the alcohol and acid, heat to boiling-point, leave to cool, leave for some days standing with frequent agitation, filter.

Use as the old tincture, the objects being prepared and washed out with 50 per cent. alcohol. Mayer only recommends it as a *succedaneum* of paracarmine.

Since this fluid contains in itself all the necessary salts (§ 248), it gives good results with *all classes of objects*.

CHAPTER XV

HÆMATEIN (HÆMATOXYLIN) STAINS

277. Introduction. *Hæmatoxylin* is a dye extracted from logwood. It is a substance that oxidises very readily, thus becoming converted into *hæmatein*, or, as often happens, into other more highly oxidised products. It appears to be now thoroughly well established (see NIETZKI, *Chemie der organischen Färbstoffe*, Berlin, Springer, 1889, pp. 215—217, and MAYER, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 170) that the colouring agent in solutions of logwood or hæmatoxylin is not the hæmatoxylin itself, but hæmatein formed in them (or, in some cases, one of the higher oxidation products).

Hæmatein is an acid body, a "colour acid" (§§ 233, 237). Substantively employed, it is a very weak plasma stain. But combined with appropriate mordants it becomes basic, and can be made to give a powerful nuclear stain, or at the same time a nuclear and a selective plasma stain. The mordants employed in histology are aluminium, chromium, iron, copper, and (rarely) vanadium and molybdenum. Aluminium and iron salts are the mordants most employed, the former furnishing lakes used for progressive staining of material in bulk, the latter forming in most cases in the tissues a lake that requires differentiation, and is only applicable to the staining of sections.

The presence of a sufficient amount of hæmatein in staining solutions was formerly brought about by allowing solutions of hæmatoxylin to oxidate spontaneously by exposure to air. The change thus brought about in the solutions is known as "ripening," and until it has taken place the solutions are not fit to use for staining.

It was discovered by MAYER and UNNA independently (see MAYER in *Mitth. Zool. Stat. Neapel*, x, 1891, pp. 170—186 ; UNNA in *Zeit. wiss. Mik.*, viii, 1892, p. 483) that nothing is easier than to bring about this change artificially ; all that is necessary being, for instance, to add to a solution of hæmatoxylin containing alum a little neutralised solution of peroxide of hydrogen or other powerful oxidising agent.* The solution becomes almost instantaneously dark blue, "ripe" and fit for staining. Other methods

* Reintroduced (*Zeit. wiss. Mik.*, xxix, 1912, p. 69) by PIAZZA, who adds to Boehmer's solution about 20 per cent., to Delafield's about 7 per cent., to Ehrlich's about 12 per cent. of peroxide of hydrogen.

of "ripening," or of preparing hæmatein separately, are given further on, and constitute a great progress (§§ 315, 316, etc.). For under the old practice of leaving staining solutions to "ripen" by the action of the air, it is necessary to wait for a long time before the reaction is obtained. During all this time, it may be weeks or months, there is no means, except repeated trial, of ascertaining whether the solution at any moment contains sufficient hæmatein to afford a good stain. And here a second difficulty arises: the oxidising process continuing, the solutions become "over-ripe"; the hæmatein, through further oxidation, passes over into colourless compounds, and the solutions begin to precipitate. They are therefore, in reality, a mixture in constantly varying proportions of "unripe," "ripe," and "over-ripe" constituents (the first and last being useless for staining purposes), and, in consequence, their staining power is very inconstant.

Logically, therefore, as concluded by Mayer, not hæmatoxylin, but *hæmatein*, should be taken in the first instance for making the staining solution.

But this is not always indicated; for such solutions may easily over-oxidise, either in the bottle or on contact with the tissues. So that it is *always* preferable to start from hæmatoxylin. In this case, it should not be done by dissolving the hæmatoxylin straight away in the other ingredients of the staining solution. The solutions should be made up from a strong *stock solution* made by dissolving hæmatoxylin crystals in absolute alcohol: one in ten is a good proportion. This solution should be kept for a long time—months, at least, a year if possible; it gradually becomes of a vinous red, and should not be used till it has become quite dark. It has then become to a great extent oxidised into hæmatein, and the staining solutions made up from it will be at once fairly ripe.

Hæmatein (or hæmatoxylin) affords a stronger stain than carmine, and gives better results with tissues fixed in osmic or chromic mixtures. The alum solutions are indicated for staining in bulk, iron hæmatoxylin for sections.

278. Hæmatoxylin is found in commerce in the form of crystals, either colourless or brownish, easily soluble in either water, glycerin or alcohol. The brownish crystals are to be preferred since the unbleached dye keeps better in solutions. In America, only the certified dye should be bought. Should difficulty be experienced in getting good selectivity with the American product, McClung (*Science,* 58, 1923, p. 515) recommends adding 3 drops of a saturated solution of lead acetate to 100 c.c. of a $\frac{1}{2}$ per cent. solution of hæmatoxylin and then shaking. After standing some hours, a black precipitate is formed. After filtration, a bright clear liquid remains which should stain satisfactorily.

HANCE (*Science*, 77, 1933, p. 287) recommends the addition of a little sodium bicarbonate to freshly prepared hæmatoxylin solution, noting that the staining and keeping properties of the solution are greatly improved.

279. Hæmatein is found in commerce as a brown powder, entirely, though with difficulty, soluble in distilled water and in alcohol, giving a yellowish-brown solution, which remains clear on addition of acetic acid. Alkalies dissolve it with a blue-violet tint. (See also *previous editions*.)

280. Iron Hæmatoxylin, Generalities.* This method is due to BENDA (*Verh. Phys. Ges.*, 1885—1886, Nos. 12, 13, 14; *Arch. Anat. Phys.*, 1886, p. 562; *third ed.* of this work, p. 365).

The method was independently worked out about the same time by M. HEIDENHAIN. The method is almost universally practised in the form given by Heidenhain, not on account of any essential difference between the two, for there is none, but chiefly because Heidenhain has given more precise instructions concerning the process.

After carefully comparing Heidenhain's process with Benda's later process (next §), we find that the two give an absolutely identical stain; that is to say, that if you mordant in Benda's *liquor ferri* (next §), and differentiate in the same, you will get exactly the same effect as by mordanting in ferric alum and differentiating in the same. But you may vary the results somewhat by varying the differentiation. Benda has pointed out (*Verb. Anat. Ges.*, xv, 1901, p. 156) that you may differentiate either by an agent which simply dissolves the lake—such as acetic or hydrochloric acid; or by an oxidising agent, such as chromic acid, or the *liquor ferri* or the ferric alum. The former, he thinks, are the best for the demonstration of nuclear structures, the latter for cytoplasmic structures. For these he greatly recommends WEIGERT's borax-ferricyanide mixture, as being the easiest and safest to employ.

We find that differentiation in the iron salt (§ 281 or § 282) is sufficient for almost all purposes. Acetic acid of 30 per cent. acts much too quickly to be safe, and causes swelling of the tissues.

VAN GIESON's picro-säurefuchsin has been recommended as a differentiation fluid by Benda (*Deutsch. med. Wochenschr.*, 1898, No. 30). We find it gives very delicate differentiations, but acts very slowly, requiring nearly as many hours as the iron alum solutions does minutes. The addition of the säurefuchsin to the picric acid is, we find, not necessary, and may prove an injurious complication.

In these processes hæmatoxylin is generally used for the stain, *not hæmatein*, the iron salt oxidising it into hæmatein, or into a

* See also §§ 627, 693, 1365.

higher oxidation product. We have obtained some good stains with hæmatein, but also some very bad ones; presumably the solutions easily over-oxidise on contact with the iron salt.

The hæmatoxylin is generally dissolved in water. LEE frequently prefers alcohol, of 50 per cent., as less injurious to tissues.

The method is a regressive one. It has been proposed to stain progressively, which we have tried, and have had extremely bad results.

The differentiation requires to be carefully timed. For this reason the method is only applicable to *sections*, which should be thin, best not over 10 μ.

Iron hæmatoxylin is one of the most important of stains. It enables us to stain elements which cannot be selectively stained in any other way. The stain is very powerful, and of a certain *optical quality* that is peculiarly suited to the employment of high powers; it will allow of the use of higher eye-pieces than other stains. It will take effect on any material, and is quite permanent. Further details as to the characters of the stain are given in § 282.

281. BENDA'S **Later Iron Hæmatoxylin** (*Verb. d. Anat. Ges.*, vii, 1, 1893, p. 161). Sections are mordanted for twenty-four hours in *liquor ferri sulphurici oxidati*, P.G.,* diluted with one or two volumes of water. They are then well washed, first with distilled water, then with tap water, and are brought into a 1 per cent. solution of hæmatoxylin in water, in which they remain till they have become thoroughly black. They are then washed and differentiated. The differentiation may be done either in 30 per cent. acetic acid, in which case the progress of the decoloration must be watched, or in a weaker acid, which will not require watching; or in the sulphate solution strongly diluted with water.

We find that if the iron solution be taken for the differentiation, it should be taken *extremely* diluted (of a *very pale* straw-colour, about 1 : 30 of water), and the progress of the differentiation watched; as if it be only diluted about tenfold, for instance, the decoloration is extremely rapid. See also last §.

Lee found that Benda's mordant is unnecessarily, sometimes harmfully, strong, and that the *liquor ferri* may be diluted *tenfold* with advantage. The duration of the bath in the mordant is also for most purposes excessive as directed by Benda. We find that three to six hours in the solution diluted tenfold is generally sufficient with favourable material.

* This preparation consists of sulphate of iron, 80 gms.; water, 40; sulphuric acid, 15; and nitric acid, 18, and contains 10 per cent. of Fe. Doubtless the *ferri persulphatis liquor* B. P. will do instead; the point is, to have a per-salt, and not a proto-salt.

282. HEIDENHAIN'S **Iron Hæmatoxylin** * (M. HEIDENHAIN, "Uber Kern und Protoplasma," in *Festschr. für Kölliker*, 1892, p. 118). Sections are treated from half an hour to at most two or three hours with a 1·5 to 4 per cent. solution of ferric alum (ammonio-ferric sulphate). By this is always meant in histology the double salt of *ammonium* and sesquioxide of iron $(NH_4)_2Fe_2(SO_4)_4$, in clear violet crystals; the double salt of the protoxide, or salt of MOHR in green crystals, will not serve. If the crystals have become yellow and opaque, they have gone bad, and should be rejected. They ought to be kept in a stoppered bottle, and the solution should be made in the cold (*Arch. mik. Anat.*, xliii, 1894, pp. 431, 435). The sections are then washed with water and stained for half an hour in an aqueous solution (of about 0·5 per cent.) of hæmatoxylin. They are then rinsed with water, and again treated with the iron solution, which slowly washes out the stain. The progress of the differentiation ought to be controlled under the microscope. The sections should to this end be removed from time to time from the alum solution, and put into tap-water whilst they are being examined. This is favourable to the stain. As soon as a satisfactory differentiation has been obtained, the preparations are washed for at least a quarter of an hour in running water, but not more than an hour, and mounted. The results differ according to the duration of the treatment with the iron and the stain. If the baths have been of short duration, viz. not more than half an hour in the iron and as much in the stain, *blue* preparations will be obtained. These show a very intense and highly differentiated stain of nuclear structures, cytoplasmic structures being pale. If the baths in the iron and in the stain have been prolonged (twelve to eighteen hours), and the subsequent differentiation in the second iron bath also duly prolonged, *black* preparations will result. These show chromosomes stained, central corpuscles stained intensely black, cytoplasm sometimes colourless, sometimes grey, in which case achromatic spindle-fibres and cell-plates are stained, connective-tissue fibres black, red blood-corpuscles black, micro-organisms sharply stained, striated muscle very finely shown.

Later (*Zeit. wiss. Mik.*, xiii, 1896, p. 186) Heidenhain gives further instructions for the employment of this stain in the study of central corpuscles. All alcohol should be removed from the tissues by means of distilled water before bringing them into the mordant. This should be a 2½ per cent. solution of ferric alum, *not weaker*. Leave the sections therein (fixed to slides by the water method, § 208) for six to twelve hours, or at least not less than three. Keep the slides upright in the mordant, not lying flat. Wash out *well* with water before staining. Stain in a "ripened" hæmatoxylin solution, *i.e.* one that has stood for four

* See also §§ 627, 693, 1365.

weeks [of course, if you make it up with the ripened brown alcoholic solution recommended § 277 *sub fin.*, this will be superfluous]. Stain from twenty-four to thirty-six hours. *Use the same staining solution over and over again* until it becomes spoilt; for the solution after having been used gives a more energetic stain, owing to its containing a trace of iron brought over by the sections. Differentiate in a 2½ per cent. solution of ferric alum. Rinse for ten minutes in running water, clear with xylol, *not* with any essential oil, and mount in xylol-balsam. See also under " Centrosomes," and " Chromosomes," etc.

Bielaszewics (*Bull. Acad. Cracovie,* 1909, 2 serié, p. 152) differentiates with very weak solution of calcium chloride; Guarnieri (*Mon. Zool. Ital.*, xvii, 1906, p. 44) with saturated solution of picric acid.

Gurwitsch (*Zeit. wiss. Mik.*, xviii, 1902, p. 291) floods sections on the slide with mordant, warms on a water-bath till bubbles are given off or the mordant becomes turbid, then stains with the hæmatoxylin in the same way. The whole process takes about ten minutes.

Held (*Arch. Anat. Phys., Anat. Abth.*, 1897, p. 277) adds to the staining bath a very little of the iron-alum solution until a scarcely perceptible precipitate is produced. A dangerous practice. Lee found it is not even safe to add a little of an over-used solution (*supra*).

Francotte (*Arch. Zool. Expér.*, vi, 1898, p. 200) mordants with *tartrate* of iron, Mallory (*Journ. Expér. Med.*, v, 1900, p. 15) with chloride.

283. Iron Hæmatoxylin (Bütschli, *Unters. uber mikroskopische Schsume u. das Protoplasma*, etc., 1892, p. 80). Sections treated with a weak brown aqueous solution of ferric acetate, washed with water, and stained in 0·5 per cent. aqueous solution of hæmatoxylin. A stain of extraordinary intensity, used by Bütschli for sections, 1 μ in thickness, of Protozoa.

284. Weigert's Iron Hæmatoxylin Mixture (*Zeit. wiss. Mik.*, xxi, 1904, p. 1). Mix 1 part of a 1 per cent. solution of hæmatoxylin in alcohol of 96 per cent. with 1 of a solution containing 4 c.c. of *liq. ferri sesquichlor.*, 1 c.c. of officinal hydrochloric acid (sp. gr. 1·124) and 95 of water. The mixture may be kept for some days (until it begins to smell of ether), but is *best used fresh.* Stain sections for a few minutes; no differentiation is necessary.

For an earlier process of Weigert's (*Allg. Zeit. Psychiatr.*, 1894, p. 245) see *last edition.*

Morel and Bassal (*Journ. Anat. Phys.*, xlv, 1909, p. 632) stain *in bulk* in Weigert's mixture with the addition of 1 c.c. of 4 per cent. solution of acetate of copper.

285. Janssens' **Iron Hæmatoxylin** (**"Hematoxyline noire"**; *La Cellule*, xiv, 1897, p. 207). A similar mixture to that of Delafield, ferric alum being taken instead of ammonia alum, the rest as in Delafield's. A progressive stain, nuclear: for yeast cells.

286. Hansen's **Iron Hæmatoxylin** (*Zeit. wiss. Mik.*, xxii, 1905, p. 55). A solution of 10 grm. ferric alum in 150 c.c. water is added to a solution of 1·6 grm. hæmatoxylin in 75 c.c. water, the mixture heated to boiling-point and cooled without access of air. Filter before use. To get a pure nuclear stain, add dilute sulphuric acid.

287. Aluminium Hæmatein (Alum Hæmatoxylin) Generalities. The mordant and dye are generally combined in a single staining

bath, giving a *progressive* stain. The stain is in different tones of blue or red according to the composition of the staining solution. Neutral or alkaline solutions give a blue stain; acid solutions give a red one. In order to *get a blue stain* in preparations that have come out red through the acidity of the staining bath, it is a common practice to treat them with weak ammonia, in the belief that the blue colour is restored by neutralisation of the acid that is the cause of the redness. According to MAYER, the ammonia acts, not by neutralising the acid, but by precipitating the alumina, which carries down the hæmatein with it (if no alumina were present the colour would be purple, not blue). The *same result* can generally be obtained by merely washing out with common tap-water, which is usually sufficiently alkaline, and can be obtained with certainty by treatment with bicarbonate of soda or acetate of soda or potash. And this is the preferable course, as ammonia is certainly a dangerous thing to treat delicate tissues with. See SCOTT's tap-water substitute, § **1429** *bis*. Of course this is a different question from that of *neutralising* with an alkali tissues that have been treated with an acid to correct over-staining. Here the neutralisation may be indicated in the interest of the *preservation* of the stain.

SQUIRE (*Methods*, p. 22) finds that sections can be blued in a few seconds by treatment with a 1 : 1000 solution of bicarbonate of soda in distilled water. MAYER holds that acetate of potash is the most inoffensive reagent to take; a strength of 0·5 to 1 per cent. may be taken.

Several of these solutions have a great tendency to over-stain. Over-staining may be corrected by washing out with weak acids (*e.g.* 0·1 to 0·2 or even 0·5 per cent. of hydrochloric acid, or with oxalic or tartaric acid), but this is not favourable to the permanence of the stain. CARNOY (*La Cellule*, xii, 2, 1897, p. 215) recommends iodised water. If acids be used, it is well to neutralise afterwards with ammonia or bicarbonate of soda (0·1 per cent.).

Bicarbonate of soda may be used for neutralisation with 70 per cent. *alcohol* as the vehicle (VON WISTINGHAUSEN, *Mitth. Zool. Stat. Neapel*, x, 1891, p. 41).

Over-staining may be avoided by staining very slowly in dilute solutions. The purest chromatin stains are obtained by staining for a short time (sublimate sections half an hour, say) in solutions of *medium* strength, such as hæmalum diluted ten to twenty-fold with water. The stain obtained either with very strong solutions, or with the slow stain of the dilute solutions, is at the same time a plasma-stain, which of course may or may not be desired. MAYER says that very dilute solutions will give a pure nuclear stain if they have been diluted with *alum-solution*, or have been *acidified*. Chrome-osmium material will not yield a pure chromatin stain unless it is very *fresh*; it is consequently

next to impossible to obtain the reaction with paraffin sections of such material; they constantly give a plasma-stain in addition to the chromatin stain, which is not the case with sublimate material.

The stain is fairly permanent in balsam, but is very liable to fade a little, and may fade a great deal. If acids have been used after staining, great care should be taken to wash them out thoroughly before mounting. In aqueous media the stain cannot be relied on to keep (this refers to the old solutions: MAYER finds that his hæmatein preparations have kept well for at least some months in glycerin, if not acid, and, with certain precautions, in balsam). Turpentine-balsam should not be used.

Formulæ §§ 288 to 299 give *aqueous* solutions, and §§ 300 to 303 *alcoholic* ones.

288. MAYER'S **Hæmalum, Later Formula** (*Zeit. wiss. Mik.*, xx, 1903, p. 409). *Hæmatoxylin*, 1 grm.; water, 1 litre. Dissolve and add 0·2 grm. of iodate of sodium ($NaIO_3$) and 50 grm. of alum, dissolve and filter.

This is an amended formula. The original one (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 172) was: 1 grm. of *hæmatein* (or the ammonia salt, §§ 278, 279) dissolved with heat in 50 c.c. of 90 per cent. alcohol, and added to a solution of 50 grm. of alum in a litre of distilled water.

This solution does not keep very well, but may be made more stable by adding 50 grm. of chloral hydrate and 1 grm. of citric (or acetic) acid.

It stains equally well, *either at first, or later*. Concentrated, it stains sometimes almost instantaneously, or in any case very rapidly. (Spring water or tap-water containing lime must not be used for diluting; perhaps weak solution of alum in distilled water is the best.) After staining, sections may be washed out either with distilled or tap water. It is *admirable for staining in bulk*. Large objects will, however, require twenty-four hours' staining, and should be washed out for the same time (this should be done with 1 per cent. alum solution if a sharp nuclear stain be desired). All alum must be carefully washed out of the tissues before mounting in balsam; and it is well to blue the stain with tap-water or otherwise, § 1429 *bis*. The stain is generally a nuclear one; in any case such may be obtained by washing out with alum-solution. Mayer's preparations have kept well in glycerin (care being taken not to have it acid), also in balsam. If oil of bergamot be used for clearing, it must be thoroughly removed by means of oil of turpentine before mounting, and oil of cloves is dangerous. It is best (Mayer, *in litt.*) to use only xylol, benzol, or chloroform, and to mount in xylol-balsam or chloroform-balsam or benzol-balsam.

Hæmalum may be mixed with alum-carmine, Säurefuchsin, or

the like, to make a double staining mixture; but it seems preferable to use the solutions in succession.

289. Mayer's **Acid Hæmalum** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 174). This is hæmalum with 2 per cent. glacial acetic acid (or 4 per cent. common acetic acid). To be used as the last, washing out with ordinary water in order to obtain a blue-violet tint of stain. The solution keeps better.

290. Unna's **Half-ripe Constant Stock Solution** (*Zeit. wiss. Mik.*, viii, 1892, p. 483).

Hæmatoxylin	1 gm.
Alum	10 gm.
Alcohol	100 c.c.
Water	200 c.c.
Sublimed sulphur	2 gm.

If the sulphur be added to the hæmatoxylin solution only when the latter has become somewhat strongly blue, *i.e.* after two or three days' time, the stage of oxidation attained by the solution will be fixed for some time by the sulphur, and according to Unna the solution will remain "constant" in staining power. Mayer (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 309) finds that the sulphur process does not preserve the solutions for long, whilst *glycerin* does. See below, "Glychæmalum."

291. Mayer's **Glychæmalum** (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 310). Hæmatein (or hæmateate of ammonia), 0·4 grm. (to be rubbed up in a few drops of glycerin); alum, 5 grm.; glycerin, 30; distilled water, 70. The stain is *not purely nuclear*, but may be made so by washing out with alum solution or a weak acid. The solution *keeps admirably*.

Rawitz (*Leitfaden*, 2nd ed., p. 63) takes 1 grm. hæmatein, 6 grm. ammonia alum, 200 grm. each of water and glycerin.

Or (*Zeit. wiss. Mik.*, xxv, 1909, p. 391) 1 grm. hæmatein, 10 grm. of nitrate of aluminium, 250 grm. each of water and glycerin.

292. Hansen's **Solution** (*Zool. Anz.*, 1895, p. 158). See *fourth edition*.

293. Harris's **Solution** (*Micr. Bull.*, xv, 1898, p. 47; *Journ. App. Mic.*, iii, p. 777). Alum-hæmatoxylin solution ripened by addition of mercuric oxide. Mayer (*Grundzüge*, 1901, p. 171) finds the formula "gives too much hæmatein."

294. **Böhmer's Hæmatoxylin** (*Arch. mik. Anat.*, iv, 1868, p. 345; *Aerzt. Intelligenzbl., Baiern.*, 1865, p. 382). Make (A) a solution of hæmatox. cryst. 1 gm., alcohol (absolute) 12 c.c., and (B) alum 1 g.m., water 240 c.c. For staining, add two or three drops of A to a watch-glassful of B.

The alcoholic solution of hæmatoxylin ought to be *old and dark* (§ 277).

A. G. Hornyold (*Trans. Manch. Micr. Soc.*, 1915) prepares:—Solution A: hæmatoxylin, 0·7 grm.; absolute alcohol, 20 c.c. Solution B: alum, 0·35 grm.; aq. dest., 60 c.c. Mix A and B, expose to light in window for three or four days, then add 20 drops of tincture of iodine. Stain sections five to ten minutes till red-brown. Differentiate in 70 per cent. alcohol, to which add a few drops of acetic acid. The sections then turn blue. Good stain after osmic fixatives.

295. Delafield's Hæmatoxylin (*Zeit. wiss. Mik.*, ii, 1885, p. 288; frequently attributed erroneously to GRENACHER or PRUDDEN). To 400 c.c. of saturated solution of ammonia-alum (that is about 1 to 11 of water) add 4 grm. of hæmatox. cryst. dissolved in 25 c.c. of strong alcohol. Leave it exposed to the light and air in an unstoppered bottle for three or four days. Filter, and add 100 c.c. of glycerin and 100 c.c. of methylic alcohol (CH_4O). Allow the solution to stand (uncorked) until the colour is sufficiently dark, then filter.

According to NEILD (*Science*, 79, 1934, p. 209) the ripening process may be shortened to an afternoon, if the alum and hæmatoxylin solution is exposed to the rays of a Cooper-Hewitt burner in a shallow dish for an hour, and then, after adding the other ingredients, the solution is exposed for two hours more.

This solution keeps for years. It is well to allow it to ripen for at least two months before using it.

For staining, enough of the solution should be added to pure water to make a very dilute stain. It is an extremely powerful stain.

It is still much used. We find that when *well ripened*—for years rather than months—it is quite a first-class stain.

BÜTSCHLI (*Unters. üb. mikroscopische Schäume u. das Protoplasma*, etc., 1892) recommends, under the name of "acid hæmatoxylin," solution of Delafield very strongly diluted, and with enough acetic acid added to it to give it a decidedly red tint. This gives a sharper and more differentiated nuclear stain than the usual solution.

MARTINOTTI (*Zeit. wiss. Mik.*, xxvii, 1910, p. 31) makes it up with 0·2 per cent. of *hæmatein*, and less alum (2 per cent.).

296. Ehrlich's Acid Hæmatoxylin (*Zeit. wiss. Mik.*, 1816, p. 150). Water 100 c.c., absolute 100, glycerin 100, glacial acetic acid 10, hæmatoxylin 2 grm., alum in excess.

Dissolve the hæmatoxylin in the alcohol, then add the acid, then the glycerine and water.

Let the mixture ripen in the light (with occasional admission of air) until it acquires a dark red colour. It will then *keep*, with constant power, *for years*, if kept in a well-stoppered bottle. It is very appropriate for staining in bulk, as over-staining does not occur. We find it excellent.

MANN (*ibid.*, xi, 1895, p. 487) makes up this stain with an equal quantity of hæmatein instead of hæmatoxylin.

MAYER (*Gründzüge*, LEE and MAYER, 1st ed., p. 154) finds that this is too much and makes the mixture overstain; 0·4 grm. of hæmatein is quite enough.

297. BURCHARDT's Pyroligneous Acid Hæmatoxylin (*Arch. mik. Anat.*, liii, 1898, p. 232) would seem to be superfluous.

298. UNNA'S **Oxidised Hæmatoxylin** (from MARTINOTTI, *Zeit. wiss. Mik.*, xxvii, 1910, p. 31). Hæmatoxylin 0·5, alum 2, water 60, alcohol 10, glycerin 20, peroxide of hydrogen solution 10, carbonate of soda 0·05.

MARTINOTTI, *loc. cit.*, makes it up with *hæmatein* (0·2 grm.).

299. APÁTHY'S **Hæmatein Mixture I A** (*Mitth. Zool. Stat, Neapel*, xii, 1897, p. 712). Make (A) a solution of 9 per cent. alum, 3 per cent. glacial acetic acid, and 0·1 per cent. salicylic acid in water, and (B) a 1 per cent. solution of hæmatoxylin in 70 per cent. alcohol, preserved for from six to eight weeks in a bottle not quite full. Mix 1 part of A with 1 of B and 1 of glycerin. Stains either sections or material in bulk. Apáthy uses it for staining neuro-fibrils.

300. KLEINENBERG'S **Hæmatoxylin** (*Quart. Journ. Micr. Sci.*, lxxiv, 1879, p. 208). Highly irrational and very inconstant in its composition and its effects; see early editions; also the criticism of MAYER (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 174), and that of SQUIRE in his *Methods and Formulæ*, p. 25, and the alternative formulæ of SQUIRE (*loc. cit.*) and of VON WISTINGHAUSEN (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 41).

301. MAYER'S **Hæmacalcium** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 182). Hæmatein (or hæmateate of ammonia, §§ 278, 279), 1 grm.; chloride of aluminium, 1 grm.; chloride of calcium, 50 grm.; glacial acetic acid, 10 c.c. (or common acetic acid, 20 c.c.); 70 per cent. alcohol, 600 c.c. Rub up finely together the first two ingredients, add the acid and alcohol, dissolve either cold or with heat; lastly add the chloride of calcium.

If the objects stain in too red a tone they may be treated with a solution (of about 2 per cent.) of chloride of aluminium in 70 per cent. alcohol, or with a 0·5 to 1 per cent. solution of acetate of soda or potash in absolute alcohol; but washing with neutral alcohol will generally suffice.

With certain objects this solution does not penetrate well. This may be remedied by acidifying the solution, or, which is better, by leaving the objects for some time before staining in acid alcohol. Anyway objects ought NOT to have an alkaline reaction. If these precautions be taken, it will not be necessary to use acid for washing out.

The solution is not recommended as giving as good results as hæmalum, and Mayer recommends it merely as a substitute for Kleinenberg's in cases in which an *alcoholic* hæmatein stain seems indicated, as being easy to prepare, and constant in its effects.

302. MAYER'S **Hæmastrontium** (*Grundzüge*, LEE and MAYER, 1910, p. 166). One gramme hæmatein, 1 grm. aluminium chloride, 50 grm. strontium chloride, 600 c.c. alcohol of 70 per cent., and (if desired) 0·25 grm. citric acid. Prepare and use as hæmacalcium.

303. De Groot's **Alcoholic Hæmalum** (*Zeit. wiss. Mik.*, xxix, 1912, p. 182). Mix 20 c.c. of glycerin with 240 of alcohol of 70 per cent. Take 4 c.c. of the mixture, 2 c.c. of hydrogen peroxide, and 0·5 grm. of hæmatoxylin, and dissolve with heat. Add 60 c.c. of the mixture, 4 grm. of calcium chloride, and 2 grm. of sodium bromide. Dissolve, add 3 grm. of alum, heat and add 100 c.c. of the mixture. When the alum is dissolved add 0·2 grm. of ferri-cyanide of potassium; dissolve and add 3 grm. more of alum and the rest of the mixture. Said to stain almost as well as hæmalum. Wash out with alcohol of 70 per cent.

304. **Other Alumina-Hæmatein Solutions.** A large number of suppressed receipts will be found given in the *earlier editions.*

305. R. Heidenhain's **Chrome Hæmatoxylin** (*Arch. mik. Anat.*, xxiv, 1884, p. 468, and xxvii, 1886, p. 383). Stain for twelve to twenty-four hours in a $\frac{1}{3}$ per cent. solution of hæmatoxylin in distilled water. Soak for the same time in a 0·5 per cent. solution of neutral chromate of potash. Wash out the excess of chromate with water.

Objects that have been fixed in corrosive sublimate ought to be very carefully washed out with iodine, or the like, as neutral hæmatoxylin forms a black precipitate with any excess of sublimate that may remain in the tissues. See Tornier, in *Arch. mik. Anat.*, 1886, p. 181.

The process is *adapted to staining in bulk.* You can decolour the objects to any extent by prolonging the soaking in the chromate. Bichromate will do instead of the neutral chromate.

306. Apáthy's **Modification of Heidenhain's Process** (*Zeit. wiss. Mik.*, v, 1888, p. 47). This is an *alcoholic* method. Stain in a 1 per cent. solution of hæmatoxylin in 70 or 80 per cent. alcohol. Differentiate sections of 10 to 15 μ, half the time of staining, sections of 25 to 40 μ twice the time of staining, in 1 per cent. solution of bichromate of potash in 70 to 80 per cent. alcohol, and wash out in alcohol of 70 per cent. All these processes should be done *in the dark.*

For celloidin series of sections, Apáthy (*ibid.*, 1889, p. 170) stains in the hæmatoxylin solution as above for ten minutes; then removes the excess of hæmatoxylin fluid from the sections by means of blotting-paper, and brings the series for from five to ten minutes into 70 per cent. alcohol containing only a few drops of a strong (5 per cent.) solution of bichromate.

307. Schultze's **Chrome Hæmatoxylin** (*Zeit. wiss. Mik.*, xxi, 1904, p. 5). The tissues to be fixed for twelve or more hours in a bichromate or chromic acid solution, preferably an osmium-bichromate mixture or liquid of Flemming, then to be washed out for twenty-four hours in 50 per cent. alcohol in the dark and stained for twenty-four hours or more in 0·5 per cent. hæmatoxylin in alcohol of 70 per cent., then washed out in alcohol of 80 per cent.

308. Hansen's **Chrome Hæmatoxylin** (*ibid.*, xxii, 1905, p. 64). Ten grm. of chrome alum boiled in 250 c.c. of water till green, and 1 grm. hæmatoxylin (dissolved in 15 c.c. of water) added; to the mixture when cold add 5 c.c. of sulphuric acid of 10 per cent. and (drop by drop) a solution of 0·55 grm. of bichromate of potash in 20 c.c. of water. Filter before use. Wash out with water free from air.

309. **Vanadium Hæmatoxylin** (Heidenhain, *Enzyk. mik. Technik.*, 1903, p. 518). Add 60 c.c. of a 6 per cent. solution of hæmatoxylin to a 0·25 per cent. solution of vanadate of ammonium (quantity not stated; should be 30 c.c., see Cohn in *Anat. Hefte*, xv, 1895, p. 302). The mixture to be used after three or four days; it will not keep over eight days. To be used with sections of sublimate material. A strong *plasma* stain for special purposes, especially mucous glands.

310. BENDA'S **Copper Hæmatoxylin** (*Arch. mik. Anat.*, xxx, 1887, p. 49). See *fourth edition.* According to our experience, not to be compared with iron hæmatoxylin, and superfluous.

311. MALLORY'S **Phospho-molybdic Acid Hæmatoxylin** (*Anat. Anz.*, 1891, p. 375). One part 10 per cent. phospho-molybdic acid solution, 1 part hæmatoxylin, 100 parts water, and 6 to 10 parts chloral hydrate. Let the solution ripen for a week in sunlight, and filter. *Chiefly* for central nervous system. Sections should be stained for from ten minutes to one hour, and washed out in two or three changes of 40 to 50 per cent. alcohol. It is necessary that the solution should be *saturated* with hæmatoxylin in order to obtain the best results ; if a good stain be not obtained at once, more hæmatoxylin must be added. Water must never be used for diluting it.

See also RIBBERT (*Centralb. allg. Path.*, vii, 1896, p. 427 ; *Zeit. wiss. Mik.*, xv, 1898, p. 93), PATELLANI (*Mon. Zool. Ital.*, xiii, 1902, p. 6), and GOLOVIN (*Zeit. wiss. Mik.*, xix, 1902, p. 184).

SARGENT (*Anat. Anz.*, xv, 1898, p. 214) quotes this stain, preceded by mordanting for twenty-four hours in 5 per cent. sulphate of copper, as KENYON'S.

KODIS (*Arch. mik. Anat.*, lix, 1901, p. 211) takes hæmatoxylin, 1 part; molybdic anhydride, 1·5 ; water, 100 ; H_2O_2, 0·5, or a crystal of HgO.

POLICE (*Arch. Zool. Napoli*, iv, 1909, p. 300) takes 0·35 grm. hæmatoxylin, 10 drops phospho-molybdic acid of 10 per cent., 10 grm. chloral hydrate, and 100 grm. alcohol of 70 per cent.

312. MALLORY'S **Phospho-tungstic Hæmatoxylin** (*Journ. Exp. Med.*, v, 1900, p. 19 ; *Zeit. wiss. Mik.*, xviii, 1901, p. 178) :

Hæmatoxylin	0·1 gm.
Water	80·0 c.c.
10 per cent. solution of (MERCK'S) phospho-tungstic acid	20·0 c.c.
Peroxide of hydrogen (U.S. Ph.) . .	0·2 c.c.

(Dissolve the hæmatoxylin, add the acid, then the peroxide.) Stain sections from two to twenty-four hours, wash out with water. A *polychromic* stain, nuclei blue, intercellular substances pink. We consider this a fine stain.

313. DONAGGIO'S **Tin Hæmatoxylin** (*Ann. Nevrol. Napoli*, xxii, 1904, p. 192). A 1 per cent. solution of hæmatoxylin is poured slowly into an equal volume of 20 per cent. solution of pink-salt (ammonia-chloride of tin). Keep in the dark.

314. **Osmium Hæmatoxylin.** SCHULTZE (*Zeit. wiss Mik.*, xxvii, 1910, p. 465) treats tissues for twenty-four hours or more with osmic acid of 1 per cent., washes well with water, and puts for a couple of days into ripened 0·5 per cent. solution of hæmatoxylin in alcohol of 35 to 50 per cent. Wash out for a day or more with alcohol of 70 per cent. Intense plasma stain.

315. J. ANDERSON'S **Rapid Method of Ripening Hæmatoxylin with Hypochlorite** (*Journ. Path. and Bact.*, 1923) :

Stock Solutions

A. Saturate boiling distilled water with ammonia (or potash) alum. Allow to cool and crystallise for twenty-four hours. Filter.

B. Shake up 2 grm. of commercial "chloride of lime" in 100 c.c. of distilled water. Allow to stand for four hours with occasional shaking. Filter. Or use a 2 per cent. solution of chloramine T.

Shake up 0·25 grm. of hæmatoxylin in 5 c.c. of absolute alcohol, add 20 c.c. solution B. Mix for a few seconds. Add this dark brown solution to 70 c.c. of solution A with constant shaking. Add 5 c.c. glacial acetic acid.

The hæmatoxylin is then ready for use. If it becomes purplish or bluish add more acetic acid. The stain that has been used may be filtered back into the bottle. It is not usually necessary to filter the stain on to the slide. This hæmatoxylin stains very rapidly; two to three minutes is usually ample.

316. H. E. Shortt's **Rapid Method for Heidenhain Stain** (*Ind. Jour. Med. R.*, 1923). The procedure is as follows :—

To 95 c.c. of distilled water in a flask one adds 1 grm. of pure hæmatoxylin crystals (Grübler for preference). This solution is slowly brought to the boiling-point, with occasional shaking to complete solution of the hæmatoxylin, and at this stage 5 c.c. of pure carbolic acid, liquefied if necessary, are added.

The solution is now allowed to cool and is then ready for use. Extensive trial, under varied conditions of temperature and climate, of solutions so prepared has shown that this stain is absolutely reliable in use, and the addition of the carbolic acid seems to increase the power of penetration so that by following the usual technique an intense nuclear stain is obtained with unfailing regularity.

317. Held's Molybdic Acid Hæmatoxylin, see § 815.

CHAPTER XVI

PLASMA STAINS * WITH COAL-TAR DYES

318. Introduction. By a plasma stain is meant one that stains the extra-nuclear parts of cells and the formed material of tissues, or one of these.

The plasma stains described in this chapter are for the most part those obtained by means of " acid " dyes (§ 237); but some of them are obtained by means of " neutral " dyes (§ 237), and a few by " basic " dyes.

The mode of staining is generally progressive, almost always so when acid colours, used substantively, are employed. But the regressive method, with differentiation, is sometimes made use of, especially when a mordant has been used with the dye.

In some processes, *e.g.* Flemming's orange method, a basic and an acid dye (or *vice versâ*) being employed *in succession*, there is formed *in the tissues* a neutral colour (§ 237) which effects the desired stain. These may be considered as adjective stains, the first colour serving as a mordant for the second. Not any two dyes taken at haphazard will behave in this way: they must be such as to form by combination a *suitable* neutral lake (*cf.* § 237). The basic dye may be made the primary stain, as in Flemming's process: or the contrary.

In such stains as Reinke's orange method, or the Ehrlich-Biondi mixture, and many others, one or more neutral colours are formed *in the mixture* and stain progressively.

Excepting Biebrich scarlet, we are not acquainted with any plasma stain that is thoroughly satisfactory for delicate work. In addition to Biebrich scarlet, we recommend for sections Säurefuchsin, either alone or in the form of Ehrlich-Biondi mixture, or Ehrlich's triacid: for material in bulk, picric acid (but only for rough work).

319. Säurefuchsin (Acid Fuchsin, Fuchsin S, Acid Rubin, Rubin S, Saurerubin, Acid Magenta, Magenta S). This must not be

* This chapter includes only such stains as are used in *ordinary* work on tissues in bulk or sections, stains for *special* purposes being treated under " Nervous tissue," " Blood," etc. It includes some double or triple stains that colour nuclei as well as plasma, but in different hues. Refer to the British dye index of the Society of Dyers and Colourists (1923) or Schultz's " Farbstofftabellen " (1923). For most purposes Dr. H. J. Conn's book, " Biological Stains " (1936) will give all information needed.

confounded with basic fuchsin, as seems to have been done by some writers.

This dye is highly soluble in water, less so in alcohol. Use a 0·5 per cent. solution in water and allow it to act on sections for a few minutes in the case of easily stainable material, or twenty-four hours or more for chrome-osmium material. The stain is fast to neutral alcohol. It is very sensitive to alkalies, so that overstains can easily be removed by washing for a few minutes in tap-water. Acids strengthen the stain, so that it is frequently useful to treat sections after staining for a few seconds with acidulated water. A good stain should show the cytoplasm, together with nuclear spindles and asters, stained red, and connective tissue strongly brought out. It may be advisable to acidify the staining bath *very slightly*. Successful stains are admirably sharp.

320. Pyronin. A basic dye, red, only used in mixtures.

According to Conn (*op. cit.*) these are Pyronin G, now difficult to obtain, and Pyronin B, which can be used quite well instead of Pyronin G in Pappenheim's stain described below. Solubility of Pyronin B at 26° C., in water 0·07 per cent., in alcohol 1·08 per cent.

Pappenheim (*Arch. Path. Anat.*, clxvi, 1901, p. 427) takes 2 parts 1 per cent. solution of methyl green and 1 part 1 per cent. solution of pyronin, stains sections for five minutes, rinses and differentiates in a solution of resorcin or hydroquinon in absolute alcohol. According to Corti and Ferrara, *Mon. zool. Ital.*, xvi, 1905, p. 319, this mixture generally stains chromatin green and cytoplasm red, but in Flemming or Hermann material the reverse. It seems to us a *coarse* plasma stain, but likely to be sometimes useful.

Unna's carbol-pyronin-methyl green modification (*Enzyk. Mik. Tech.*, 1910, ii, p. 412 : Lee was indebted for the formula to Dr. Gaudlitz) is as follows : Stain for five to ten minutes at 30° to 40° C. in methyl green 0·15 parts, pyronin 0·25, alcohol 2·5, glycerin 20, and carbolic acid of 0·5 per cent. to make up 100 volumes. Cool rapidly, rinse, dehydrate, and pass through bergamot oil, or xylol or benzol (*not clove-oil*), into balsam. Brings out bacteria (red) in organic liquids.

321. Orange G. This is the benzazo-beta-naphthol-disulphonate of soda. As indicated by its chemical description, this is an " acid " colour.

Solubility at 26° C., in water 10·86 per cent., in alcohol 0·22 per cent.

It is easily soluble in water, less so in alcohol. Use as directed for Säurefuchsin. Almost, if not quite, as precise a stain as Säurefuchsin. It does not overstain, but may wash out other dyes.

In recent years this stain has been much used as a counterstain after iron alum hæmatoxylin. Saturate absolute alcohol and pass the slides through in their way to xylol or benzol balsam.

SÄUREFUCHSIN AND ORANGE G. Lee has had good results by mixing the aqueous solutions of these two dyes, but unfortunately has not noted the proportions. SQUIRE (*Methods and Formulæ*, p. 42) takes 1 grm. Säurefuchsin, 6 grm. Orange G in 60 c.c. of alcohol and 240 c.c. of water. See also under "Connective tissues."

322. EHRLICH-BIONDI **Mixture** (or EHRLICH-BIONDI-HEIDENHAIN Mixture) (*Pfluger's Arch.*, xliii, 1888, p. 40).

To 100 c.c. saturated aqueous solution of orange add with continual agitation 20 c.c. saturated aqueous solution of Säurefuchsin (Acid Fuchsin) and 50 c.c. of a like solution of methyl green.

(According to Krause (*Arch. mik. Anat.*, xlii, 1893, p. 59), 100 parts of water will dissolve about 20 of Säurefuchsin (Rubin S), 8 of orange G and 8 of methyl green.) The solutions must be *absolutely saturated*, which only happens after several days.

Dilute the mixture with 60 to 100 volumes of water. The dilute solution ought to redden if acetic acid be added to it; and if a drop be placed on blotting-paper it should form a spot bluish-green in the centre, orange at the periphery. If the orange zone is surrounded by a broader red zone, the mixture contains too much fuchsin.

According to M. HEIDENHAIN ("Ueber Kern u. Protoplasma," in *Festchr. f. Kolliker*, 1892, p. 115) the orange to be used should be "Orange G," the Acid Fuchsin or Säurefuchsin should be "Rubin S" ("Rubin" is a synonym of Fuchsin) and the methyl green should be "Methylgrün OO." And Lee thought it *absolutely necessary* that these ingredients be those prepared under those names by the *Actienfabrik für Anilinfabrikation in Berlin.*

The strong solutions directed to be taken readily precipitate on being mixed. To avoid this it is recommended by Squire (*Methods and Formulæ*, etc., p. 37) to dilute them before mixing.

Other proportions for the mixture have been recommended by KRAUSE (*loc. cit. supra*), viz. 4 c.c. of the Säurefuchsin solution, 7 of the orange G and 8 of the methyl green; the mixture to be diluted 50 to 100-fold with water. THOME (*Arch. mik. Anat.*, lii, 1898, p. 820) gives the proportions 2 : 5 : 8, and dilutes 100-fold.

Stain sections (N.B. *sections only*) for six to twenty-four hours. Dehydrate with alcohol, clear with xylol, and mount in xylol balsam.

In the intention of the observers who have elaborated this stain it is a *progressive* stain, and *not* a regressive one. It does not require any differentiation, and the sections should be got

through the alcohol into xylol as quickly as possible in order to avoid any extraction of the methyl green, which easily comes away in the alcohol. Drüner (*Jena Zeit.*, xxix, 1894, p. 276) stains for ten minutes in the *concentrated* solution, treats for one minute with alcohol containing 0·1 per cent. of hydrochloric acid, and then with neutral alcohol.

The best results are obtained with *sublimate material;* chrome-osmium material, and the like, give a much inferior stain. Preparations made with the usual mixture, as given above, are liable to fade; by *acidifying* the mixture a stronger and more sharply selective stain is obtained, which does not fade. But too much acid must not be added. According to the *Enzyk. mik. Technik,* you may add 15 to 24 drops of 0·2 per cent. acetic acid to 100 c.c. of the diluted solution.

Another process of acidification is given by M. Heidenhain (*Ueber Kern und Protoplasma*, p. 116); for this see *fourth edition.* See also Israel (*Prakticum Path. Hist.*, 2 Aufl., Berlin, 1893, p. 69); Trambusti (*Ricerche Lab. Anat. Roma*, v, 1896, p. 82; *Zeit. wiss. Mik.*, xiii, 1896, p. 357); and Thome (*op. cit. supra*). Eisen (*Proc. Calif. Acad.* (3), i, 1897, p. 8) acidifies with oxalic acid.

After acidification the solution must not be filtered, and if it has been kept for some time a little more acid must be added.

Before staining (M. Heidenhain, *loc. cit.*), sections should be treated for a couple of hours with 0·1 per cent. acetic acid, then for ten to fifteen minutes with officinal tincture of iodine, and be rinsed with alcohol before bringing into the stain. The treatment with acid is necessary in order to ensure having the sections *acid* on mounting in balsam. The primary object of the iodine is to remove any sublimate from the preparations, but it also is said to enhance the power of staining of the chromatin with methyl green, and to produce a more selective staining of protoplasmic elements.

The stain is a very fine one when successful. But it is *very capricious.* The correct result should be a precise chromatin stain combined with a precise stain of the cytoplasm by the Säurefuchsin. Now the least defect or excess of acidity causes the plasma stain of the Säurefuchsin to become a diffuse one, instead of being sharply limited to the plastin element. It is difficult to dehydrate the sections without losing the methyl green. For this reason the stain will only work with very thin sections; to be quite sure of good results, the sections should be of not more than 3 μ in thickness, and if they are over 5 the desired results are almost hopeless. The stain keeps very badly. Lee stated that the method has its *raison d'être* for the very special objects for which it was imagined—for the researches on cell-granulations for which Ehrlich employed the three colours, or for the researches on the ground cytoplasm for which Martin Heidenhain employed the mixture; for the study of gland cells; and for similar objects. But to recommend it, as has been done, as a general stain for ordinary work, is nothing but mischievous exaggeration. For it is far from having the qualities that should be possessed by a normal section stain. Workers have at length found this out, and it is now but little used except for the special purposes above indicated.

323. Ehrlich's **"Triacid" Mixture.** This name would seem to indicate that the mixture contains three "acid" colours,

which is not the case, methyl green being a strongly "basic" colour. Ehrlich explains in a letter to MAYER (see also EHRLICH and LAZARUS, *Die Anæmie*, 1898, p. 26) that it is so called "because in it all the three basic groups of the methyl green are combined with the acid dye-stuffs." A very pretty conundrum!

The latest receipt (*op. cit.*, p. 28) is as follows:

Prepare separately saturated solutions of orange G, Säurefuchsin, and methyl green, and let them clarify by settling. Then mix, in the order given, using the same measure-glass, 13 to 14 c.c. of the orange, 6 to 7 of the Säurefuchsin, 15 of distilled water, 15 of alcohol, $12\frac{1}{2}$ of the methyl green, 10 of alcohol, and 10 of glycerin. After adding the methyl green, shake well, but do not filter.

The mixture keeps well. Lee found its qualities and defects to be much those of the Ehrlich-Biondi mixture. The stain seems more powerful but less delicate, and the methyl green in it appears to have more resistance to alcohol, so that it is better adapted for ordinary work.

MAYER (*Grundzüge*, LEE and MAYER, p. 197) has simplified the formula thus: Take 1 g. methyl green, 2 g. orange, 3 g. Säurefuchsin, and dissolve in a mixture of 45 c.c. water, 10 c.c. glycerin, and 20 c.c. alcohol of 90 per cent.

MOREL and DOLERIS (*C. R. Soc. Biol.*, liv, 1902, p. 1255) mix 1 vol. of the solution with one of 8 per cent. formalin and add 0·1 per cent. of acetic acid, and state that thus the methyl green is better fixed in the nuclei.

324. PIANESE'S Säurefuchsin-malachite Green (from MÜLLER, *Arch. Zellforsch.*, viii, 1912, p. 4) consists of 0·5 grm. malachite green, 0·1 grm. Säurefuchsin, and 0·01 grm. Martius yellow in 150 c.c. water and 50 c.c. alcohol. Stain for twenty-four hours, differentiate with alcohol, containing 1 to 2 drops of HCl per 200 c.c.

325. Picric Acid. Picric acid gives useful plasma stains after carmine and hæmatoxylin. The *modus operandi* consists merely in adding picric acid to the alcohols employed for dehydrating the objects.

Solubility at 26° C., in water 1·18 per cent., in alcohol 8·96 per cent.

Picric acid has considerable power of washing out other anilin stains; and *in combinations with hydrochloric acid it very greatly enhances* the power with which this acid washes out carmine stains. It should, therefore, not be added to the acidulated alcohol taken for differentiating borax-carmine stains, or the like, but only to the neutral alcohol used afterwards. It has the great quality that it can be used for staining *entire objects*, and is much indicated for such objects as small Arthropods or Nematodes, mounted whole.

It can in some cases be employed by dissolving it in the solution of another dye (see Picro-carmine, LEGAL's alum-carmine, § 256, etc.); or (for sections) by dissolving it in the xylol or chloroform used for clearing.

Though picric acid is a useful ground stain, it is at most a *rough* one, being *very diffuse*. It stains, however, horn, chitin, muscle and erythrocytes, with special energy.

According to FROHLICH (*Zeit. wiss. Mik.*, xxvii, 1910, p. 349) *picraminic acid* (from Grübler & Hollborn) has some advantages over picric acid.

326. VAN GIESON'S **Picro-Säurefuchsin** (from *Zeit. wiss. Mik.*, xiii, 1896, p. 344). To a saturated aqueous solution of picric acid are added a few drops of saturated aqueous solution of Säurefuchsin, until the mixture has become garnet-red. Or (*Trans. Amer. Micr. Soc.*, xix, 1898, p. 105) to 100 parts of the picric acid solution add 5 parts of 1 per cent. solution of Säurefuchsin. After staining (section only), rinse with water, dehydrate, and clear in oil of origanum.

OHLMACHER (*Journ. Exper. Med.*, ii, 1897, p. 675) adds 0·5 per cent. of Säurefuchsin to a saturated solution of picric acid which has been diluted with an equal quantity of water. He uses this after previous staining with gentian violet.

RAMÓN Y CAJAL recommends 0·1 grm., of Säurefuchsin to 100 of saturated solution of picric acid (SCHAFFER, *Zeit. wiss. Zool.*, lxvi, 1899, p. 236).

HANSEN (*Anat. Anz.*, xv, 1898, p. 152) adds 5 c.c. of 2 per cent. solution of Säurefuchsin to 100 c.c. saturated solution of picric acid, and for staining adds to 3 c.c. of the mixture one-third of a drop of 2 per cent. acetic acid, stains for a few minutes or hours, rinses in 3 c.c. of water with 2 drops of the acidified stain added, dehydrates clears with xylol, and mounts in xylol-balsam. Connective-tissue red, elastin and all other elements yellow.

WEIGERT (*Zeit. wiss. Mik.*, 1904, p. 3) adds 10 parts of 1 per cent. Säurefuchsin to 100 of saturated picric acid.

See also MÖLLER, *op. cit.*, xv, 1898, p. 172.

This stain is generally used as a contrast stain to follow hæmatoxylin. APÁTHY (*Behrens' Tabellen*, 3rd ed., p. 129) takes for this purpose 1 grm. of Säurefuchsin in 500 c.c. of saturated solution of *picrate of ammonia*.

WILHELMI (*Fauna Flora Golf. Neapel*, xxii, 1909, p. 18) takes 0·2 grm. Säurefuchsin, 0·8 grm. picrate of ammonia, 10 grm. absolute alcohol, and 89 grm. water.

E. and T. SAVINI (*Zeit. wiss. Mik.*, xxvi, 1909, p. 31) use a formula due to BENDA. Ninety-five volumes of saturated solution of picrate of ammonia are mixed with 5 volumes of 1 per cent. solution of Säurefuchsin. For use, two to four drops of saturated solution of picric acid are added to 10 c.c. of the mixture. This neither overstains nor attacks the primary stain.

327. FLEMMING'S **Orange Method** * (*Arch. mik. Anat.*, xxxvii, 1891, pp. 249 and 685). Stain sections *of Flemming or Hermann material* in strong alcoholic safranin solution diluted with anilin water (§ 372); differentiate in absolute alcohol, containing at most 0·1 per cent. of hydrochloric acid, until hardly any more colour comes away; stain for one to three hours in gentian violet (§ 373); wash for a short time in distilled water; treat with concentrated, or at least fairly strong,

* Refer also to § 814.

aqueous solution of orange G. After at most a few minutes, whilst pale violet clouds are still being given off from the sections on agitation, bring them into absolute alcohol until hardly any more colour comes away, clear in clove or bergamot oil, and mount in damar or balsam before the last pale clouds of colour have ceased to come away. The orange *must* be *orange G.*

WINIWARTER and SAINMONT (*Zeit. wiss. Mik.*, xxv, 1908, p. 157, and *Arch. Biol.*, xxiv, 1909, p. 15) stain for twenty-four hours in the gentian, wash out after the orange for two to three hours in 100 c.c. absolute alcohol with 3 to 4 drops of HCl, and differentiate finally with oil of cloves.

This is not a triple stain in the sense of giving three different colours in the result; it is a nuclear and plasmatic stain in mixed tones; the orange, apparently, combines with the gentian to form a "neutral" dye, soluble in excess of the orange, which thus differentiates the stain.

See also FLEMMING in *Arch. Anat. Phys. Anat. Abth.*, 1897, p. 175.

328. REINKE'S **Orange Method** (*Arch. mik. Anat.*, xliv, 2, 1894, p. 262). To a concentrated aqueous solution of gentian violet are added "a few drops" of a like solution of orange G. The solution precipitates in part, owing to the formation of an imperfectly soluble "neutral" colour, but becomes almost clear again if an excess of water be added. The solution is not to be filtered, but the sections are to be stained in the mixture made almost clear by addition of water. It is said that the "neutral" solution may be preserved for future use by adding to it one-third of alcohol. After staining (sections previously stained with safranin), you differentiate rapidly with alcohol and clear with clove oil.

Lee has tried this process and obtained exactly the same results as with Flemming's process, and so have other workers.

ARNOLD'S **Orange Method** (*Arch. Zellforsch.*, iii, 1909, p. 434). Sections (of chrome material) are treated for five minutes with solution of equal parts of iodine and iodide of potassium in alcohol of 40 per cent., then washed and stained for four hours in a saturated solution of safranin in alcohol of 75 per cent.: then washed and put for five to fifteen minutes into solution of 7 parts of methylen blue, 0·5 of carbonate of soda and 100 of water, washed, dehydrated, and treated until pale blue with solution of orange G in oil of cloves. Cytoplasmic reticulum blue on orange ground, nucleoli and centrosomes red. Instead of the safranin, basic fuchsin may be taken.

329. BONNEY'S **Triple Stain** (*Virchow's Arch.*, cxciii, 1908, p. 547). Stain sections (of acetic alcohol or sublimate material, not chrome or formol material) for two minutes in a solution of 0·25 parts methyl violet and 1 part pyronin in 100 of water. Wipe slide dry, and flood twice with the following: 2 per cent. aqueous solution of orange G, boiled and filtered, is added drop by drop to 100 c.c. of acetone, with agitation, until there is formed a flocculent precipitate, which redissolves on further addition of the orange. Wash rapidly in pure acetone, and pass through xylol into balsam. Chromatin violet, cytoplasm red, connective-tissue yellow, keratin violet. Not adapted for blood films.

330. **Bordeaux R.** An "acid" dye, giving a general stain taking effect both on chromatin and cytoplasm, and a very good plasma stain. Lee used for chrome-osmium material a

1 per cent. solution, and stained for twelve to twenty-four hours. The stain is sufficiently fast.

Solubility at 26° C., in water 5·18 per cent., in alcohol 1·12 per cent.

331. Bordeaux R, Thionin, and Methyl Green (GRÄBERG, *Zeit. wiss. Mik.*, xiii, 4, 1896, p. 460).

332. Congo Red (Congoroth) (see GRIESBACH, in *Zeit. wiss. Mik.*, iii, 1866, p. 379). An " acid " colour. Its solution becomes blue in presence of the least trace of free acid (hence Congo is a valuable reagent for demonstrating the presence of free acid in tissues; see the papers quoted *loc. cit.*). A stain much of the same nature as Säurefuchsin. It is useful for staining some objects during life. CARNOY (*La Cellule*, xii, 1897, p. 216) has had very good results with it after hæmatoxylin of DELAFIELD. He used 0·5 per cent. solution in water. Note that this colour is not to be confounded with other Congos, as Congo yellow, or brilliant Congo. It is one of the azo dyes.

333. Congo-Corinth. Also an acid dye. HEIDENHAIN (*Zeit. wiss. Mik.*, xx, 1903, p. 179) recommends Congo-Corinth G (or the allied colour **Benzopurpurin 6 B**) (Elberfelder Farbwerke). Sections *must be made alkaline* before staining, by treating them with very weak sal ammoniac or caustic soda, in alcohol. After staining, pass through absolute alcohol into xylol. Used after alum hæmatoxylin, the stain of which it does not cause to fade.

334. Benzopurpurin. According to GRIESBACH (*loc. cit.*, § 332), another " acid " colour very similar in its results to Congo red. See also ZSCHOKKE (*ibid.*, v. 1888, p. 466), who recommends **Benzopurpurin B**, and says that weak aqueous solutions should be used for staining, which is effected in a few minutes, and alcohol for washing out. **Delta-purpurin** may be used in the same way.

See last § as to the necessity of *alkalising* the sections, which Heidenhain states is necessary with all dyes of this group.

335. Neutral Red (Neutralroth) (EHRLICH, *Allg. med. Zeit.*, 1894, pp. 2, 20; *Zeit. wiss. Mik.*, xi, 1894, p. 250; GALEOTTI, *ibid.*, p. 193). A " basic " dye. The term " neutral " refers to the hue of its solution. Its neutral red tint is turned bright red by acids, yellow by alkalies. The stain in tissues is in general metachromatic, nuclei being red, cell-bodies yellow (*cf.* ROSIN, in *Deutsche med. Wochenschr.*, xxiv, 1898, p. 615; *Zeit. wiss. Mik.*, xvi, 2, 1899, p. 238). Up to the present this colour has chiefly been employed for *intra-vitam* staining. Tadpoles kept for a day or two in a solution of 1 : 10,000 or 100,000 absorb so considerable a quantity of the colour that they appear a dark red. The stain is limited to cytoplasmic granules (EHRLICH), and to the contents of mucus cells (GALEOTTI). See also §§ 680, and 766.

Solubility at 26° C., in water 5·64 per cent., in alcohol 2·45 per cent.

According to EHRLICH and LAZARUS (*Spec. Pathol. und Therapie*, herausgeg. von NOTHNAGEL, viii, 1, 1898, p. 1; *Zeit. f. wiss Mik.*,

xv, 3, 1899, p. 338) it may be used for *intra-vitam* staining of tissues in the same way as methylen blue, by injection or immersion with contact of air. It is especially a *granule* stain. Similar results are recorded by ARNOLD (*Anat. Anz.*, xvi, 1899, p. 568, and xxi, 1902, p. 418). See also EHRLICH and LAZARUS, *Anæmie*, i, 1898, p. 85; LOISEL (*Journ. de l'Anat. et de la Physiol.*, 1898, pp. 197, 210, 217) (*intra-vitam* staining of sponges); and PROWAZEK (*Zeit. wiss. Zool.*, lxii, 1897, p. 187) (*intra-vitam* staining of Protozoa). We have had very good results with it as an *intra-vitam* stain.

It has been found useful for staining, in hardened material, the corpuscles of NISSL (*q.v.*) in nerve-cells. S. MAYER (*Lotos*, Prague, 1896, No. 2) states that it also stains degenerating myelin. The solutions that have been employed for staining fixed material are strong aqueous ones, 1 per cent. to concentrated. (See also §§ 766 *et seq.*).

336. Biebrich Scarlet (BRITISH DYES, Ltd., Huddersfield). A. K. GORDON (*British Medical Journ.*, 1917, p. 828) finds this an excellent acid dye which never overstains and is not diffuse. Use in 1 per cent. solution, or as recommended by SCOTT.

We have used this stain a good deal and find it better than any other plasma dye that we have tried.

337. The Eosins, found in commerce under the names of **Eosin, Saffrosin, Primerose Soluble, Phloxin, Bengal Rose, Erythrosin, Pyrosin B, Rose B, à l'Eau,** etc., are all " acid " phthalein colours. They are not quite identical in their properties. Most of them are soluble both in alcohol and in water, but some only in alcohol (" *Primerose a l'Alcool* ").

According to CONN (*op. cit.*) the proper dye is ethyl eosin, and when ordering supplies, this name, and not alcohol soluble eosin, should be used. Solubility at 26° C., in water 0·03 per cent., in alcohol 1·13 per cent.

They are all *diffuse* stains, formerly much used as contrast stains, less so now. HANSEN (*Anat. Hefte*, xxvii, 1905, p. 620) adds 1 drop of acetic acid of 2 per cent. to 9 c.c. of 1 per cent. eosin, which makes the stain more selective.

Eosin is a stain for red blood-corpuscles, and also for certain granules of leucocytes (see under " Blood ").

The yolk of some ova takes the stain strongly, so that it is useful in some embryological researches.

338. For **Bengal Rose** see GRIESBACH, *Zool. Anz.*, 1883, p. 172.

339. EHRLICH'S **Indulin-Aurantia-Eosin,** or **Acidophilous Mixture,** or **Mixture C,** or **Mixture for Eosinophilous Cells** (from the formula kindly sent LEE by Dr. GRÜBLER). Indulin, aurantia, and eosin, of each 2 parts; glycerin, 30 parts. This gives a very quick, syrupy solution. To use it, coverglass preparations may

be floated on to it; or sections on slides may have a few drops poured on to them, the slide being laid flat till the stain has taken effect (twenty-four hours for Flemming material). Lee found that with Flemming material it gives a powerful and good stain, which is much more resistant to alcohol than that of the EHRLICH-BIONDI mixture, and is, therefore, much more adapted to ordinary work. The stain keeps well.

ISRAEL (*Praktik. Path. Hist.*, Berlin, 1893, p. 68) gives a more complicated receipt.

340. Methyl Green and Eosin (CALBERLA, *Morph. Jahrb.*, iii, 1877, Heft 3, p. 625; LIST, *Zeit. wiss. Mik.*, ii, 1885, p. 147; BALBIANI, *Ann. Microgr.*, Paris, vii, 1895, p. 245; RHUMBLER, *Zeit. wiss. Zool.*, lxi, 1895, p. 38). See early editions.

341. Methylen Blue and Eosin (CHENZINSKY, quoted from *Zeit. wiss. Mik.*, xi, 2, 1894, p. 269).

Methylen blue, sol. sat. in water . . .	40 c.c.
Eosin, 0·5 per cent. in 70 per cent. alcohol .	20 c.c.
Distilled water, or glycerin	40 c.c.

This solution will only keep for about eight days.

PIANESE (*ibid.*, xi, 1894, p. 345) adds a considerable proportion of carbonate of lithium.

See also the mixture of BREMER (*Arch. mik. Anat.*, xlv, 1895, p. 446).

Lee has tried CHENZINSKY'S mixture as a tissue stain, without good results; but see ROSIN, *Berliner klin. Wochenschr.*, 1898, p. 251; *Zeit. wiss. Mik.*, xvi, 1899, p. 223, and xvii, 1900, p. 333.

See also LAURENT (*Centralb. allg. Path.*, xi, 1900, p. 86; *Zeit. wiss. Mik.*, xvii, 1900, p. 201).

342. MALLORY'S Eosin and Methylen Blue (*Journ. Med. Research*, January, 1904). Sections of ZENKER material (other sublimate material not so good) are stained for half to three-quarters of an hour at 56° C. in 5 per cent. aqueous solution of eosin, rinsed and flooded with solution of 1 part of methylen blue, and 1 of potassium carbonate in 100 of water, diluted with about 7 parts of water. After forty minutes they are flooded (not washed) with water, and differentiated for about five minutes in alcohol of 95 per cent. Absolute alcohol, xylol, balsam.

343. Other Eosin and Methylen-blue Stains. For some very important ones see under "Blood."

344. Light Green (Lichtgrun S. F.). An "acid" colour, soluble in alcohol, and a good plasma stain.

Solubility at 26° C., in water 20·35 per cent., in alcohol 0·82 per cent.

BENDA (*Verh. physiol. Ges. Berlin*, December 18th, 1891, Nos. 4 u. 5) stains sections for twenty-four hours in anilin-water safranin solution, then for about half a minute in a solution of 0·5 grm. Lichtgrün or Säureviolett (Grübler) in 200 c.c. of alcohol dehydrates and mounts in balsam. This process gives a very elegant stain, but requires very thin sections, and there is always risk of the safranin being washed out. The Lichtgrün stain unfortunately does not keep at all well.

See also PRENANT, *Arch. mik. Anat.*, vii, 1905, p. 430, and

GUIEYSSE, *C.R. Soc. Biol.*, lxii, 1907, p. 1212, and BAILEY (*Jour. Med. Res.*, 1920).

345. Janus Green (MICHAELIS, *Arch. mik. Anat.*, lv, 1900, p. 565). Used in solution of 1 : 30,000 for staining mitochondria (pancreas, salivary glands, etc.) in the fresh state. For method of using refer to § 761, p. 339.

Solubility at 26° C., in water 5·18 per cent., in alcohol 1·12 per cent.

346. Malachite Green (syn. **Solid Green, Victoria Green, New Green, Benzoyl Green, Fast Green**). A basic colour, which has been used as a plasma stain for the ova of *Ascaris* by VAN BENEDEN and NEYT. These authors used it for glycerin preparations ; it can hardly be got into balsam.

FLEMMING (*Arch. mik. Anat.*, xix, 1881, p. 324) attributes to it a special affinity for nucleoli.

347. Iodine Green ("HOFMANN'S Grün"), see GRIESBACH (*Zool. Anz.*, No. 117, vol. v, 1882, p. 406). Stain essentially that of methyl green, but plasma often violet through the presence of a violet impurity (MAYER, *Mitth. Zool. Stat. Neapel*, xii, 1896, p. 311 ; see also earlier editions). It is now only used by botanists.

348. Thiophen Green (Thiophengrun), see KRAUSE, *Intern. Monatsschr. Anat., etc.*, iv, 1887, Heft. 2.

349. Coerulein S., a green "acid" dye, is recommended for the staining of muscle-fibrils by M. v. LENHOSSÉK (*Anat. Anz.*, xvi, 1899, p. 339). See also HEIDENHAIN, *ibid.*, xx, 1901, p. 37, and RAWITZ, *ibid.*, xxi, 1902, p. 554.

350. Quinolein Blue (Cyanin, Chinolinblau ; v. RANVIER, *Traité*, p. 102). Quinolein is said by Ranvier to have the property of staining fatty matters an intense blue.

It is useful for staining Infusoria, which in dilute solution it stains during life. See the methods of CERTES.

From the reactions mentioned by Ranvier it would seem that his "bleu de quinoléine" is not the preparation that usually goes under that name. See EHRLICH, in *Arch. mik. Anat.*, xiii, 1877, p. 266.

351. Indulin and Nigrosin. Indulin, Nigrosin, Indigen, Coupier's Blue, Fast Blue R, Fast Blue B, Blackley Blue, Guernsey Blue, Indigo substitute are the names of brands of a group of dyes, mostly "acid," related to the base violanilin. According to BEHRENS the name Indulin is generally given to a bluish brand, and that of Nigrosin to a blacker one.

Nigrosin, used with sublimate material, Lee found stains both nuclei and cytoplasm, the chromatin strongly. It will not give the stain at all with chrome-osmium material.

According to CALBERLA (*Morph. Jahrb.*, iii, 1877, p. 627) the concentrated aqueous solution of *Indulin* should be diluted with 6 volumes of water. Sections will stain in the dilute solution in from five to twenty minutes. He also says that it never stains nuclei ; the remaining cell-contents and intercellular substance are stained blue. This seems to us to be, roughly, correct.

352. Safranin and Nigrosin (or Indigo-Carmine) (KOSSINSKI, *Zeit. wiss. Mik.*, vi, 1880, p. 61). See early editions.

353. Picro-Nigrosin, PFITZER (*Deutsch. Botan. Gesellsch.*, 1883, p. 44) dissolves nigrosin in a saturated solution of picric acid in water, and uses it for fixing and staining at the same time, on the slide. See also under "Connective Tissues."

354. Anilin Blue. Under this title are comprised various "basic" derivatives of the base rosanilin. They occur under the names **Spirit Soluble Blue (Bleu Alcool), Gentian Blue 6 B, Spirit Blue O, Opal Blue, Bleu de Nuit, Blue Lumière, Parma Bleu, Bleu de Lyon.** Some authors give the name Bleu de Nuit and Gründstichblau as synonyms of **Bleu de Lyon.** The *Encycl. mik. Technik.* says it is "Anilinblau B—6 B," with many synonyms or designations of brands, Parma blue being "Anilinblau R or 2 R."

Conn (*op. cit.*) writes: "Anilin blue W.S. should be regarded as a group of dyes rather than a simple dye. The composition of the various commercial products sold under this name is uncertain." Conn mentions that "Water Blue" (see next paragraph) is also a synonym for Anilin Blue. The name "Cotton Blue" is also applied to "Methyl Blue," and "Anilin Blue."

Lee found it a fairly good stain, giving very good differentiations of nerve-tissue and of cartilage (as has already been pointed out by Baumgarten and by Jacoby). Maurice and Schulgin stain in bulk with it after borax-carmine, using a very dilute alcoholic solution. Baumgarten and Jacoby stain sections in a 0·2 per cent. alcoholic solution.

Tonkoff (*Arch. mik. Anat.*, lvi, 1900, p. 394) adds a little tincture of iodine to the solution of the dye, or mordants the sections with iodine.

Skrobansky (*Intern. Monatsschr. Anat.*, xxi, 1904, p. 20) uses it in water with picric acid.

355. Carmine Blue (**Bleu Carmin Aqueux,** from Mesiter, Lucius, and Brunig, at Höchst-a-M.). Janssens (*La Cellule*, ix, 1893, p. 9) states that this colour possesses a special affinity for the parts of cytoplasm that are undergoing cuticular differentiation. He uses it in alcoholic solution acidified.

356. Methyl Blue. Under this title are comprised some other derivatives of the base rosanilin. They are "acid" colours. Here belong **Methyl Blue, Cotton Blue, Water Blue (Wasserblau) Methyl Water-Blue, China Blue (Chinablau), Soluble Blue.** See § above.

Amongst these **Water Blue (Wasserblau)** possesses some useful properties. According to Mitrophanow (quoted from *Zeit. wiss. Mik.*, v, 1888, p. 513), used in concentrated aqueous solution it gives a very good double stain with safranin. It is very resistant to alcohol. Using the Wasserblau first, and then the safranin, Lee has had some interesting results. The Wasserblau must be used first. With chrome-osmium material, twelve to twenty-four hours in the blue, and four or five in the safranin may not be too much. Lee's stains have not kept well.

Mann (*Methods*, etc., p. 216) uses a mixture of 35 parts 1 per cent. solution of eosin, 45 of methyl blue 1 per cent., and 100 of

water. He has also (*Zeit. wiss. Mik.*, xi, 1894, p. 490) used a similar mixture for nerve-cells.

This is a famous stain. To get it right you must have the proper specimens of stains. This applies more to eosin than to the other stain. If the made-up stain is red, or blue, it is incorrect, it should be neither of these colours, but a reddish-purple.*

357. Violet B (or Methyl Violet B) (S. Mayer, *Sitzb. k. k. Akad. wiss. Wien*, iii, Abth., February, 1882). Used in solutions of 1 grm. of the colour to 300 grm. of 0·5 per cent. salt solution, and with *fresh tissues* that have not been treated with any reagent whatever, this colour is said to give a stain so selective of the elements of the vascular system that favourable objects, such as serous membranes, appear as if injected. The preparations do not keep well; acetate of potash is the least unsatisfactory medium for mounting them in, or a mixture of equal parts of glycerin and saturated solution of picrate of ammonia (*Anat. Anz.*, 1892, p. 221). See also under "Plasma-fibrils."

The allied dye, **Crystal Violet**, has been employed for staining sections, *e.g.* by Kromayer and others.

According to Conn (*op. cit.*) synonyms are: Violet C, G, or 7B, Hexamethyl violet, methyl violet 10B, gentian violet. Solubility of Conn's specimen, at 26° C., in water 1·68 per cent., in alcohol 13·87 per cent.

Benda (*Neurol. Centralb.*, xix, 1900, p. 792) stains in a mixture of 1 vol. saturated sol. of the dye in 70 per cent. alcohol, 1 vol. 1 per cent. sol. of hydrochloric acid in 70 per cent. alcohol, and 2 vols. of anilin water, the liquid being warmed until vapour is given off, then cooled and the sections dried with blotting-paper, treated one minute with 30 per cent. acetic acid, dehydrated with alcohol and cleared with xylol. Refer to § 697 for mitochondria and granules.

Kresyl Violet. An oxyazin dye, giving metachromatic stains. Herxheimer (*Arch. mik. Anat.*, liii, 1899, p. 519, and liv, p. 289) stains sections of skin with **Kresyl-echtviolett.** Nuclei blue, plasma reddish. Similarly Fick (*Centralb. allg. Path.*, xiii, 1902, p. 987; *Zeit. wiss. Mik.*, xx, 1903, p. 223), staining for three or four minutes in a concentrated aqueous solution, and differentiating in alcohol until the connective tissue has become colourless. Keratohyalin violet-red to salmon-coloured.

358. Benzoazurin may be made to give either a diffuse or a nuclear stain, according to Martin (see *Zeit. wiss. Mik.*, vi, 1889, p. 193).

359. Rawitz **"Inversion" Plasma Stains.** It has been discovered by Rawitz that by means of appropriate mordants certain basic anilins, which by the usual methods of regressive staining are pure chromatin stains, may be made to afford a pure plasma stain, thus giving an "inversion" of the usual stain. The stain is a vile one. For details see *fourth edition*, or Rawitz (*Sitzb. Gesnaturf. Freunde*, Berlin, 1894, p. 174; *Zeit. wiss. Mik.*, xi, 1895, p. 503; and his *Leitfaden f. hist. Untersuchungen*, Jena, 1895, p. 76).

360. Artificial Alizarin (Rawitz, *Anat. Anz.*, xi, 10, 1895, p. 295).

* For further information refer to § 1152.

A double stain by means of artificial Alizarin, or Alizarin-cyanin, requiring the use of special mordants supplied by the colour manufacturers, and very complicated. See *fifth edition*.

RAWITZ (*Zeit. wiss Mik.*, 1909, pp. 393 and 395) also recommends a solution of 1 grm. of **Säure-Alizarinblau BB** (or **Säuregrün G**) (both from Höchst), 10 grm. ammonia alum, 100 c.c. glycerin, and 100 c.c. water.

SZÜTZ (*ibid.*, xxix, 1912, p. 289) fixes in a mixture of 15 c.c. 1 per cent. platinum chloride, 15 c.c. formol, and 30 c.c. saturated solution of sublimate, makes paraffin sections, and stains them with Heidenhain's iron-hæmatoxylin. They are then treated for from five to six hours with 5 per cent. solution of aluminium acetate, rinsed, and stained for from five to six hours with Benda's sulphalizarinate of soda (given under "Mitochondria"), and got into balsam. A red plasma stain, affecting plasmafibrils. For *intra-vitam* stains with alizarin see FISCHEL, NILSSON, *Zool. Anz.*, xxxv, 1909, p. 196.

361. For BENDA's **Alizarin Stains,** see under "Centrosomes," "Mitochondria," and "Neuroglia," §§ 697, 1085.

CHAPTER XVII

NUCLEAR STAINS WITH COAL-TAR DYES *

362. Introduction. Very few coal-tar dyes give a precise nuclear or chromatin stain by the *progressive* method (§ 241). Two of them—methyl green and Bismarck brown—are pre-eminently progressive chromatin stains. Many of the others—for instance, safranin, gentian, and especially dahlia—may be made to give a progressive nuclear stain with fresh tissues by combining them with acetic acid; but in general are not so suitable for this kind of work as the two colours first named.

Again, very few coal-tar dyes give a pure plasmatic stain (one leaving nuclei unaffected). The majority give a diffuse stain, which in some few cases becomes by the application of the *regressive* method (§ 241) a most precise and splendid chromatin stain.

But plasma staining is generally done by the progressive method.

The basic anilin dyes were at one time greatly in vogue for the staining of chromatin in researches on the structure of nuclei. They have been little used for that purpose since the working out of the iron hæmatoxylin process, which gives a more energetic stain. But they may still be useful as a means of controlling the iron hæmatoxylin process, which frequently stains all sorts of things besides chromatin, which does not occur with the best tar colour stains.

The acid and neutral anilin dyes afford some of our best plasma stains.

We recommend—for staining nuclei of fresh tissues, methyl green; for staining nuclei of fixed tissues by the regressive method, safranin for a red stain, and gentian violet or Thionin for a blue one; as a plasma stain for sections, Säurefuchsin; for entire objects, picric acid.

A. PROGRESSIVE STAINS

363. Methyl Green. This is the most common in commerce of the "anilin" greens. It is a basic dye. It appears to go by the synonyms of *Methyl-anilin green, Grünpulver, Vert Lumière, Lichtgrün*; these two last are in reality the name of another colour. When first studied by Calberla, in 1874 (*Morphol. Jahrb.*, iii, 1887, p. 625), it went by the name of *Vert en cristaux*. It is commonly met with in commerce under the name of more costly

* For further details, see Conn, *op. cit.*

greens, especially under that of iodine green. It is important not to confuse it with the latter, nor with aldehyde green (*Vert d'Eusèbe*), nor with the phenylated rosanilins, *Paris green*, and *Vert d'Alcali, or Vèridine.*

Methyl green is the chloromethylate of zinc and pentamethly rosanilin-violet. It is obtained by the action of methyl chloride on methyl violet. The commercial dye always contains inconverted methyl violet as a consequence of defective purification. It is sometimes adulterated with anilin blue (soluble blue). It is also sometimes adulterated with a green bye-product of the manufacture—the chloride of mona-methyl-para-leukanilin. See BENEDIKT and KNECHT's *Chemistry of the Coal-tar Colours.* For tests for purity see MAYER, *Mitth. Zool. Stat. Neapel,* xii, 1896, p. 312, and FISCHER, *Fixirung, Färbung, u. Bau des Protoplasmas,* p. 89.

Methyl green is extremely sensitive to alkalies. It is therefore important to use it only in *acidified* solutions and to use only acid, or at least perfectly neutral fluids, for washing and mounting.

This is an *extremely important histological reagent.* Its chief use is as a *chromatin* stain for *fresh, unfixed tissues.* For this purpose it should be used in the form of a strong aqueous solution containing a little acetic acid (about 1 per cent. in general). *The solutions must always be acid.* If the tissues have been previously fixed with acetic acid you will not get a chromatin stain. The same applies to fixation with acetic acid sublimate; whilst pure sublimate will allow of a chromatin stain (BURCKHARDT, *La Cellule,* xii, 1897, p. 364). You may wash out with water (best acidulated) and mount in some acid aqueous medium containing a little of the methyl green in solution. The mounting medium, if aqueous, *must be acidulated.*

Employed in this way, with *fresh unfixed* tissues, methyl green is a pure chromatin stain, in the sense of being a precise colour reagent for chromatin. For *in the nucleus* it stains nothing but chromosomes or chromatin elements; it does not stain plasmatic nucleoli (unless indeed these contain chromatin), nor caryoplasm, nor achromatic filaments. *Outside* the nucleus it stains some kinds of cytoplasm and some kinds of formed material, especially glandular secretions (silk, for instance, and mucin). The chromatin elements are invariably stained a bright green (with the exception of the nuclein of the head of some spermatozoa), whilst extra-nuclear structures are in general stained in tones of blue or violet. But this metachromatic reaction is probably due to the methyl-violet impurity, and is not obtained with a chemically pure methyl green.

Staining is *instantaneous;* overstaining never occurs. The solution is very penetrating, kills cells instantly without swelling

or other change of form, and preserves their forms for at least some hours, so that it may be considered as a delicate fixative. It may be *combined without precipitating* with divers fixing or preserving agents. Osmic acid (of 0·1 to 1 per cent.) may be added to it, or it may be combined with solution of RIPART and PETIT (this is an excellent medium for washing out and mounting in).

Alcoholic solutions may also be used for staining. They also *should be acidulated with acetic acid.*

The stain does not keep easily. It is difficult to mount it satisfactorily in balsam, because the colour does not resist alcohol sufficiently (unless this be charged with the colour). The resistance of the colour to alcohol is, however (at all events if it be used in the EHRLICH-BIONDI combination), considerably increased by treating the sections for a few minutes with tincture of iodine before staining (M. HEIDENHAIN).

Of preparations mounted with excess of colour in the usual aqueous media, Lee found the most fortunate only survive for a few months. Dr. HENNEGUY, however, writes that it keeps well in BRUN's glucose medium.

Lee mentions that it was first pointed out by HESCHL (*Wiener med. Wochenschr.*, 2, 1879), that methyl green is a reagent for amyloid degeneration. His observations were confirmed by CURSCHMANN (*Virchow's Arch.*, vol. lxxix, 1880, p. 556), who showed that it colours amyloid substance of an intense violet; but this, as pointed out by SQUIRE (*Methods and Formulæ*, etc., Churchill, 1892, p. 37), is undoubtedly due to its containing methyl violet as an impurity.

364. Bismarck Brown (Manchester Brown, Phenylen Brown, Fesuvin, La Phénicienne). A fairly pure nuclear stain that will work either with fresh tissues or with such as have been hardened in chromic acid, or otherwise. It is a basic dye. Solubility at 26° C. in water 1·36 per cent., in alcohol 1·08 per cent.

The dye is not very easily soluble in water. You may boil* it in water, and filter after a day or two (WEIGERT, in *Arch. mik. Anat.*, xv, 1878, p. 258). You may add a little acetic or osmic acid to the solution. MAYSEL (*ibid.*, xviii, 1880, pp. 237, 250) dissolves the colour in acetic acid (this solution does not give a permanent stain). Alcoholic solutions may also be used, *e.g.* saturated aqueous solution diluted with one-third volume of 90 per cent. alcoholic; or CALBERLA's glycerin-and-alcohol mixture of dilute glycerin (say of 40 per cent. to 50 per cent.) may very advantageously be employed.

The watery solutions must be frequently filtered (but then much of the colour is retained on the filter). The addition to them of carbolic acid has been recommended (vide *Journ. Roy. Mic. Soc.*, 1886, p. 908). Bismarck brown stains rapidly, but

* Conn says not, as this alters the composition of the dye.

never overstains. The stain is permanent both in balsam and in glycerin.

This colour may be used as a chromatin stain for fresh tissues in the same way as methyl green. HERLA (*Arch. Biol.*, xiii, 1893, p. 423) employs for ova of *Ascaris* a mixture of 0·25 gm. vesuvin, 0·25 gm. malachite green, 10 of glycerin and 100 of water, and washes out with weak glycerin.

The chief use of this colour is for progressive staining; but it may be employed for staining by the regressive method (see § 241), and also for *intra-vitam* staining (§ 769) (for this purpose it is necessary to see that the colour employed be pure *and neutral*).

365. Other Progressive Stains. Most of the basic tar colours used for regressive staining will also give by the progressive method a nuclear stain of greater or less purity if used in solutions acidified with acetic acid. Amongst these may be mentioned *thionin*, which need not even be acidified; also, for fresh tissues especially, *gentian violet*, *dahlia*, and *toluidin blue*.

B. REGRESSIVE STAINS

366. The Practice of Regressive Staining: The Staining Bath. *Sections only*, or material that is thin enough to behave like sections, such as some membranes, can be stained by this method.

The solutions employed are made with alcohol, water, or anilin, or sometimes other fluids, according to the solubility of the colour. There seems to be no special object in making them with alcohol if water will suffice, the great object being to get *as strong a solution as possible*. Indeed, the solutions made with strong alcohol are found not to give quite such good results as those made with water or weak alcohol. Alcohol of 50 per cent. strength, however, may be said to constitute a very generally desirable medium. The sections must be *very thoroughly* stained in the solution. As a general rule they cannot be left too long in the staining fluid. With the powerful solutions obtained with anilin a few minutes or half an hour will usually suffice, but to be on the safe side it is frequently well to leave the sections twelve to twenty-four hours in the fluid. Up to a certain point the more the tissues are stained the better do they resist the washing-out process, which is an advantage. Some workers, indeed, prefer weak solutions; so HEIDENHAIN, *Enzyk. mik. Technik*, i, pp. 433, 434; but the nature of the fixing agent should be taken into account.

Material fixed in chromic or chromo-osmic mixtures gives a *sharper and more selective* stain than material fixed in sublimate or the like. In fact, to *ensure the best results, only material fixed in chromic mixtures* (or Hermann's fluid) *should be employed.*

During the staining the tissues become *overstained*, that is, charged with colour in an excessive and diffuse manner. The

stain must now be *differentiated* by removal of the excess of colour.

367. Differentiation. This is generally done with alcohol, sometimes *neutral*, sometimes *acidulated* (with HCl). The stained sections, if loose (celloidin sections), are brought into a watch-glassful of alcohol; if mounted in series on a slide, they are brought into a tube of alcohol (differentiation *can* be done by simply pouring alcohol on to the slide, but it is better to use a tube or other bath). It is in either case well to *just rinse* the sections in water, or even to wash them well in it, before bringing them into alcohol.

The sections in the watch-glass are seen to give up their colour to the alcohol in clouds, which are at first very rapidly formed, afterwards more slowly. The sections on the slide are seen, if the slide be gently lifted above the surface of the alcohol, to be giving off their colour in the shape of rivers running down the glass. In a short time the formation of the clouds or of the rivers is seen to be *on the point of ceasing;* the sections have become *pale* and somewhat *transparent*, and (in the case of chrome-osmium objects) have *changed colour*, owing to the coming into view of the general ground colour of the tissues. (Thus chrome-osmium-safranin sections turn from an opaque red to a delicate purple.) At this point the differentiation is complete, or nearly so.

It is generally directed that absolute alcohol be taken for differentiation. This may be well in some cases, but in general 95 per cent. is found to answer perfectly well. HEIDENHAIN (*Enzyk.*, i, p. 434) takes *methyl* alcohol.

The hydrochloric-acid-alcohol extracts the colour *much more quickly from resting nuclei than from kinetic nuclei.* Therefore, washing out should be done with neutral alcohol whenever it is desired to have resting nuclei stained as well as dividing nuclei; the acid process serving chiefly to *differentiate karyokinetic figures.*

The proportion of HCl with which the alcohol should be acidified for the acid process should be about 1 : 1000 or less; seldom more.

The length of time necessary for differentiating to the precise degree required varies considerably with the nature of the tissues and the details of the process employed; all that can be said is that it generally lies between thirty seconds and two minutes. The acid process is *vastly more rapid* than the neutral process, and therefore of course more risky.

There exists also a method of differentiation known as *substitution*—one stain being made to wash out another. Thus methylen blue and gentian violet are discharged from tissues by aqueous solution of vesuvin or of eosin; fuchsin is discharged from tissues by aqueous solution of methylen blue. The second stain " substitutes " itself for the first in the general " ground " of the tissues, leaving, if the operation has been

successfully carried out, the nuclei stained with the first stain, the second forming a "contrast" stain. In the paper of RESEGOTTI in *Zeit. wiss. Mik.*, v, 1888, p. 320, it is stated as a very general rule that colours that do *not* give a nuclear stain by the regressive method will wash out those that *do*. But RESEGOTTI used the second colour in *alcoholic* solution ; so that it remains uncertain how far the differentiation should be attributed to the second colour itself, and how far to the alcohol used as a vehicle. The same remark applies to BENDA'S Safranin-and-Lichtgrün process.

368. Clearing. After due differentiation, the extraction of the colour may be stopped by putting the sections into water, but the general practice is to clear and mount them at once.

You may clear with clove oil or anilin, *which will extract some more colour* from the tissues. Or you may clear with an agent that does not attack the stain (cedar oil, bergamot oil, xylol, toluol, etc. ; see the chapter on Clearing Agents). If you have used neutral alcohol for washing out, you had perhaps better clear with clove oil, as neutral alcohol does not always, if the staining have been very prolonged, extract the colour perfectly from extra-nuclear parts. But if you have not stained very long, and if you have used acidulated alcohol for washing out, clove oil is not necessary, and it may be better not to use it, as it somewhat impairs the brilliancy of the stain. A special property of clove oil is that it helps to differentiate karyokinetic figures, as it *decolours resting nuclei more rapidly than those in division.*

Some colours are much more sensitive to the action of clove oil than others ; and much depends on the quality of this much-adulterated essence. New clove oil extracts the colour more quickly than old, and anilin than clove oil.

Series of sections on slides are conveniently cleared by pouring the clearing agent over them.

After clearing you may either mount at once in damar or balsam, or stop the extraction of the colour, if clove oil have been used, by putting the sections into some medium that does not affect the stain (xylol, cedar oil, etc.). Chloroform should be avoided, either as a clearer or as the menstruum for the mounting medium.

369. General Results. The results depend in great measure on the previous treatment of the tissues. If you have given them a prolonged fixation in Flemming's *strong* chromo-aceto-osmic mixture, and have differentiated after staining with acid alcohol and cleared with clove oil, you will get, with some special exceptions, nothing stained but nucleoli and the chromatin of *dividing* nuclei, that of resting nuclei remaining unstained. If you have given a lighter fixation, with Flemming's weak mixture or some other fixing agent not specially inimical to staining, and have differentiated after staining with neutral alcohol, you will get the chromatin of *resting* nuclei stained as well. Either process may

also stain mucin, the ground-substance of connective tissues (especially cartilage), the bodies of Nissl in nerve-cells, and the yolk of ova.

370. HENNEGUY'S **Permanganate Method** (*Journ. de l'Anat. et de la Physiol.*, xxvii, 1891, p. 397). Sections are treated for five minutes with 1 per cent. solution of permanganate of potassium. They are then washed with water and stained (for about half the time that would have been taken if they had not been mordanted with the permanganate) in safranin, rubin, gentian violet, vesuvin, or the like, and are differentiated with alcohol, followed by clove oil in the usual way.

The mordanting action of the permanganate is so energetic that if it has been overmuch prolonged before staining with safranin, or, still more, with rubin, it becomes almost impossible to differentiate the sections properly ; it may be necessary to leave them for a month or more in clove oil.

371. OHLMACHER'S **Formaldehyde Process** (*Medical News*, February 16th, 1895). Ohlmacher states that formaldehyde is a powerful mordant for tar colours. Tissues may either be mordanted separately by treatment for a short time (one minute is enough for cover-glass preparations) with a 2 per cent. to 4 per cent. formalin solution ; or the formalin may be combined with the stain. One grm. of fuchsin or methylen blue dissolved in 10 c.c. of absolute alcohol may be added to 100 c.c. of 4 per cent. formalin solution. Sections are said to stain in half a minute and to resist alcohol much more than is the case with those treated by the usual solutions.

372. **Safranin.** One of the most important of these stains, on account of its power, brilliancy, and permanence in balsam, and the divers degrees of electivity that it displays for the nuclei and other constituent elements of different tissues. Solubility at 26° C. : in water 5·45 per cent., in alcohol 3·41 per cent.

The great secret of staining with safranin is *to get a good safranin.* In ordering it is well to specify whether you want it for staining muclei or for staining elastic fibres, or for what other purpose you may require it. There are presumably at least a score of sorts of safranin on the market, differing to a considerable extent in colour, weight, solubility, and histological action. Some are easily soluble in water and not so in alcohol, some the reverse, and some freely soluble in both. The brand Lee had been using for a long time, which gave good results, was the " Safranin O " of Grübler & Co.

CONN (*op. cit.*) writes: " Grübler & Co. sell four types of safranin ; safranin pur, safranin gelb, safranin O wasserlöslich, and safranin spritlöslich. The first of these is apparently methylen violet, the others are Safranin O."

Staining. The majority of safranins are not sufficiently soluble in water, so that solutions in other menstrua must be employed.

PFITZNER (*Morph. Jahrb.*, vi, p. 478, and vii, p. 291) advised a solution of safranin 1 part, absolute alcohol 100 parts, and water 200 parts, the last to be added only after a few days.

Flemming (*Arch. mik. Anat.*, xix, 1881, p. 317) used a concentrated solution in absolute alcohol, diluted with about one-half of water.

Babes (*ibid.*, 1883, p. 356) used (A) a mixture of equal parts of concentrated alcoholic solution and concentrated aqueous solution (this is very much to be recommended), or (B) a concentrated or supersaturated aqueous solution made with the aid of heat.

Some people still employ simple aqueous solutions.

The anilin solution of Babes (*Zeit. wiss. Mik.*, iv, 1887, p. 470) consists of water 100 parts, anilin oil 2 parts, and an excess of safranin. The mixture should be warmed to from 60° to 80° C., and filtered through a wet filter. This solution will keep for a month or two.

Zwaardemaker (*ibid.*, iv, 1887, p. 212) makes a mixture of about equal parts of alcoholic safranin solution and anilin water (saturated solution of anilin oil in water ;—to make it, shake up anilin oil with water and filter). This will keep for many months, perhaps indefinitely.

Lee uses equal parts of saturated solution in anilin water and saturated solution in absolute alcohol.

Differentiation.—For general directions see § 367.

Flemming's *acid* differentiation (*Zeit. wiss. Mik.*, i, 1884, p. 350). Differentiate, until hardly any more colour comes away, in alcohol acidulated with about 0·5 per cent. of hydrochloric acid, followed by pure alcohol and clove oil. (You may use the HCl in watery solution if you prefer it.) Or you may use a lower strength, viz. 0·1 per cent. at most (see *Arch. mik. Anat.*, xxxvii, 1891, p. 249) ; and this is generally preferable.

Objects are supposed to have been well fixed—twelve hours at least—in the *strong* chromo-aceto-osmic mixture, and stained for some hours. In this way you get kinetic chromatin and nucleoli alone stained.

Podwyssozki (*Beitr. z. Path. Anat.*, i, 1886, p. 289) differentiates (for from a few seconds to two minutes) in a strongly alcohol solution of picric acid, followed by pure alcohol. Same results (except that the stain will be brownish instead of pure red).

Babes recommends treatment with iodine, according to the method of Gram (see next section). This process has also been recommended by Prenant (*Int. Monatsschr. Anat.*, etc., iv, 1887, p. 368).

It has been shown by Ohlmacher (*Journ. Amer. Med. Assoc.*, vol. xx, No. 5, February 4th, 1893, p. 111) that if tissues be treated with iodine or picric acid after staining with safranin, there may be produced in the tissue elements a precipitate of a dark red substance of a crystalline nature, but of lanceolate, semilunar, falciform, or navicellar forms. The precipitate is formed both in normal and pathological tissue, readily in carcinomatous tissues ; and Ohlmacher concludes that many

of the bodies that have been described as "coccidia," "sporozoa," or other "parasites" of carcinoma are nothing but particles of this precipitate.

See also the differentiation process of MARTINOTTI and RESEGOTTI (*Zeit. wiss. Mik.*, iv, 1887, p. 328) for alcohol-fixed material, and of GARBINI (*Zeit. wiss. Mik.*, v, 2, 1888, p. 170).

In preparations made with chromo-aceto-osmic acid, safranin stains, besides nuclei, elastic fibres, the cell bodies of certain horny epithelia, and the contents of certain gland-cells (mucin, under certain imperfectly ascertained conditions).

The stain is perfectly permanent.

373. Gentian Violet* may be used in aqueous solution, or as directed for safranin. This stain is now being much used for chromosomes. Solubility at 26° C., in water 2·93 per cent., in alcohol 15·21 per cent. (crystal violet).

In some cases it may be useful to employ the method devised by GRAM for the differentiation of bacteria in tissues (*Fortschr. d. Medicin.*, ii, 1884, No. 6; *British Med. Jour.*, September 6th, 1884, p. 486; *Jour. Roy. Mic. Soc.* [N.S.], iv, 1884, p. 817). In this the sections are treated, after staining, with a solution composed of—

Iodine	1 grm.
Iodide of potassium	2 grm.
Water	300 ,,

for two or three minutes, until they become black. They are then differentiated with neutral alcohol, until they turn grey, and are then finally differentiated with clove oil.

By this process, in resting nuclei the nucleoli alone are stained, or the chromatin if stained is pale; in dividing nuclei the chromatin is stained with great intensity, being nearly black in the equatorial stage.

Gentian violet is an exceedingly powerful stain, quite as precise as safranin.

The stain keeps well. It is more or less dichroic, possibly owing to the fact that the dye is not a pure substance, but a mixture of "Krystallviolett" and methyl violet.

According to CONN (*op. cit.*) one should specify "crystal violet" for bacteriological work, or histological work where a deep blue-violet is required, or methyl violet 2 B, where in histology a reddish shade is wanted. The American Commission on Stains proposes to drop the term "Gentian Violet" at some later period.

HERMANN (*Arch. mik. Anat.*, xxxvi, 1889, p. 58) first stains for twenty-four hours or more in safranin, differentiates incompletely with alcohol, then stains for three to five minutes in the anilin-water gentian solution, treats with the iodine solution for one to three hours, and finally differentiates with absolute alcohol.

* Refer especially to § 1364.

374. Thionin. The hydrochloride of thionin, or violet of Lauth, is a colour chemically nearly allied to methylen blue. A basic dye, the solubility of which at 26° C. is in water 0·25 per cent. and in alcohol 0·25 per cent. Its action is so selective from the first that it may almost be considered to be a progressive stain. If you stain for only a short time (a few minutes) in a concentrated aqueous solution, hardly anything but the chromatin will be found to be stained. If the staining be prolonged, plasmatic elements will begin to take up the colour. After a short stain no special differentiation is required; all that is necessary is to rinse with water, dehydrate, and amount. After a strong stain you differentiate with alcohol in the usual way, with this advantage, that the stain is so highly resistant to alcohol that there is no risk whatever of over-shooting the mark; the stain will not be more extracted in an hour than gentian or dahlia is in a minute, so that the process may be controlled under the microscope if desired. For this reason this stain may be useful to beginners. It is a very powerful stain.

Thionin is a specific stain for mucin, *q.v.* Some observers have found the stain to fade. WOLFF (*Zeit. wiss. Mik.*, xv, 1899, p. 312) says that, to avoid this, preparations should be mounted in a little solid colophonium or balsam melted over a flame. FELIZAT and BRANCA (*Journ. Anat. Phys.*, xxxiv, 1898, p. 590) mount without a cover. HENNEGUY (*in litt.*) clears with acetone.

KING (*Anat. Record*, iv, 1910, p. 236) stains with a saturated solution in carbolic acid of 1 per cent., and finds the stain permanent.

NICOLLE's "thionine phéniquée" consists of 1 part of saturated solution in alcohol of 50 per cent., and 5 parts of 2 per cent. aqueous solution of carbolic acid.

375. Other Regressive Stains. The following may be useful:—

Dahlia (Hoffman Violet), according to FLEMMING (*Arch. mik. Anat.*, xix, 1881, p. 317), best used in aqueous solution, either neutral or acidified with acetic acid, and differentiated with neutral alcohol. A pure blue stain, which keeps well. See also SCHUBERG, in *Zeit. wiss. Zool.*, lxxiv, 1903, p. 7, and lxxxvii, 1907, p. 557. CONN (*op. cit.*) states that "Dahlia" is often a mixture of basic fuchsin and methyl violet. Various specimens differ very much.

Victoria Blue (Victoriablau) (Lustgarten, *Med. Jahrb. k. Ges. d. Aerzte zu Wien*, 1886, pp. 285—291). This dye ("Victoriablau 4 A") has a special affinity for *elastic fibres*. For this object Lustgarten recommends an alcoholic solution of the dye diluted with 2 to 4 parts of water. Fixation in chrome-osmium, or at least in a chromic mixture, is, we believe, a necessary condition to this reaction. And you must stain for a long time.

Victoria has also a special affinity for *mucus-cells*, from which it is not washed out by alcohol, and for cartilage.

This stain keeps well.

With **Toluidin Blue** Lee has had some superb stains of chromatin, unfortunately accompanied by a diffuse staining of cytoplasm.

MANN (*Zeit. wiss. Mik.*, xi, 1894, p. 489) states that he has had good results by staining with it after eosin.

See further, as to the micro-chemical properties of this dye, HARRIS, *The Philadelphia Medical Journal*, May 14th, 1898. It much resembles methylen blue.

METZNER (Nagel's *Handb. Phys.*, ii, 1907, p. 915) mordants sections before staining, for three-quarters of an hour in iron alum.

Magdala Red (Naphthalin Red, Rose de Naphthaline).

Fuchsin (meaning the basic fuchsins, a series of Rosanilin salts having very similar reactions, and found in commerce under the names of FUCHSIN, ANILIN RED, RUBIN, ROSEIN, MAGENTA, SOLFERINO, CORALLIN). GRASER (*Deutsche Zeit. Chirurgie*, xxvii, 1888, pp. 538—584 ; *Zeit. wiss. Mik.*, v, 1888, p. 378) stains for twelve to twenty-four hours in a dilute aqueous solution, washes out for a short time in alcohol, stains for a few minutes in aqueous solution of methylen blue, and dehydrates with alcohol. A double stain. Chromatin and nucleoli red, all the rest blue.

ZIEHL'S **Carbolic Fuchsin** (*Zeit. wiss. Mik.*, vii, 1890, p. 39) consists of basic fuchsin 1 grm., acid. carbol. crist. 5 grm., alcohol 10 grm., aq. dest. 100 grm. The stain is differentiated with alcohol followed by clove oil.

Kresofuchsin (ROTHIG, *Arch. mik. Anat.*, lvi, 1900, p. 354). Its aqueous solution is red and stains mucus, cartilage, keratin, and nuclei red, whilst its alcoholic solution is blue and stains elastin blue. See also under " Connective tissues."

Bismarck Brown has this advantage, that being sufficiently resistant to alcohol it may be utilised for staining *entire objects*.

KAISER (*Biblioth. Zool.*, H. 7, 1 Halft, 1891 ; *Zeit. wiss. Mik.*, viii, 1891, p. 363) stains for forty-eight hours, and at a temperature of 60° C. in saturated solution of Bismarck brown in 60 per cent. alcohol (the solution to be made in boiling alcohol), and washes out (until all is decoloured except the karyokinetic figures) in 60 per cent. alcohol, containing 2 per cent. hydrochloric acid or 3 per cent. acetic acid.

Methyl Violet. See *ante*, § 373.

Benzoazurin (MARTIN, *Zeit. wiss. Mik.*, vi, 3, 1889, p. 193). Stain for an hour or so in dilute aqueous solution, and wash out with HCl alcohol.

Methylen Blue.

Nigrosin (ERRERA, *Proc.-Verb. Soc. Belge de Mik.*, 1881, p. 134) gives a good stain which resists alcohol well.

Methyl Green is sometimes useful in certain mixtures (see next chapter).

CHAPTER XVIII

METHYLEN BLUE

376. Methylen Blue is a " basic " dye, being the chloride or the zinc chloride double salt of tetramethylthionin. It appears some persons have confounded it with the " acid " dye methyl blue, to which it has not, histologically, any resemblance.

Commercial methylen blue sometimes contains as an impurity a small quantity of a reddish dye, which used to be taken to be methylen red. This impurity is present from the beginning in many brands of methylen blue, is frequently developed in solutions of the dye that have been long kept (so called " ripened " solutions), and is still more frequently found in kept *alkaline* solutions. According to NOCHT (*Centralb. Bakteriol.*, xxv, 1899, pp. 764—769 ; *Zeit. wiss. Mik.*, xvi, 1899, p. 225) it is not methylen red, nor methylen violet either, but a new colour, for which NOCHT proposes the name " Roth aus Methylenblau."

CONN (*op. cit.*) says : " Methylen blue BX, B, BG, BB : grade preferred for biological work, Methylen blue U.S.P. Solubility at 26° C., in water 3·55 per cent., in alcohol 1·48 per cent. The dye is theoretically tetra-methyl-thionin."

According to MICHÆLIS (*Centralb. Bakteriol.*, xxix, 1901, p. 763, and xxx, 1901, p. 626 ; *Zeit. wiss. Mik.*, xviii, 1902, p. 305, and xix, 1902, p. 68) confirmed later by NOCHT, REUTER, and GIEMSA, this dye is **Methylenazur,** an oxidation-product of methylen blue, already described by BERNTHSEN in 1885. It is an energetic dye, of markedly metachromatic action, and to it are due the metachromatic effects of methylen blue solutions (methylen blue itself is not metachromatic).

The presence of this dye as an impurity in methylen blue is not always an undesirable factor ; on the contrary, it sometimes affords differentiations of elements of tissues or of cells that cannot be produced by any other means. Methylen blue that contains it is known as *polychrome methylen blue*, and is employed for staining *certain cell-granules*. UNNA (*Zeit. wiss. Mik.*, viii, 1892, p. 483) makes this as follows : A solution of 1 part of methylen blue and 1 of carbonate of potash in 20 of alcohol and 100 of water is evaporated down to 100 parts. (It may be used at once, or after diluting with an equal volume of anilin water, for sections, which after staining may be differentiated with glycol, creosol, or Unna's glycerin-ether mixture—all of which, as well as the polychrome methylen blue, can be obtained from Grübler. MICHÆLIS (*op. cit.*) makes it as follows : 2 gr. of medicinal methylen blue are dissolved in 200 c.c. of water, and 10 c.c. of $\frac{1}{10}$ normal solution of

caustic soda added. Boil for a quarter of an hour; after cooling add 10 c.c. of $\frac{1}{10}$ normal sulphuric acid, and filter.

Methylenazur is isolated from methylen blue by the prolonged action of an alkali or of silver oxide. It seems also that it is formed in certain mixtures of methylen blue with eosin (ROMANOWSKY, LAVERAN, GIEMSA and others), by means of the eosin, which in these mixtures acts *chemically*, and can be replaced by resorcin, hydroquinon, and the like. It is best procured from Grübler & Hollborn, who supply it pure as "Azur I," and mixed with an equal quantity of methylen blue as "Azur II." See further as to this dye under "Stains for Blood." See also an important paper by PROWAZEK (*Zeit. wiss. Mikr. Tech.*, 31).

There are several sorts of methylen blue sold, the most important being—"methylen blue, according to EHRLICH"; "methylen blue according to KOCH"; "methylen blue BX, according to S. MAYER"; "Methylenblau, medic. pur."

The colour to be employed for *intra-vitam nerve staining* should be *as pure as possible.* APÁTHY (*Zeit. wiss. Mik.*, ix, 1893, p. 466) writes that the best—in fact, the only one that will give *exactly* the results described by him—is that of E. MERCK, of Darmstadt, described as "*medicinisches* Methylenblau." DOGIEL (*Encycl. mik., Technik.*, 1st edition, p. 911) has had his best results with "Methylenblau n. Ehrlich," or "BX," obtained from Grübler & Hollborn.

377. The Uses of Methylen Blue. As a histological reagent it is used for sections of hardened central nervous tissue, in which it gives a specific stain of medullated nerves. It stains the basophilous granulations of "Mastzellen" and plasma-cells, and the granules of NISSL in nerve-cells, also mucin. It is much used—in the form of mixtures affording methylenazur—in the study of blood, blood parasites, and similar objects. For all of these see the respective sections in Part II. Further, it stains a large number of tissues *intra vitam* with little or no interference with their vital functions. And last, not least, it can be made to furnish stains of nerve tissues, intercellular cement substances, lymph spaces, and the like, that are essentially identical with those furnished by a successful impregnation with gold or silver, and are obtained with greater ease and certainty; with this difference, however, that gold stains a larger number of the nervous elements that are present in a preparation, sometimes the totality of them; whilst methylen blue stains only a selection of them, so bringing them more prominently before the eye, and allowing them to be traced for greater distances.

378. Staining *in toto* during Life. Small and permeable aquatic organisms may be stained during life by adding to the water in which they are confined enough methylen blue to give it a very light tint. After a time they will be found to be partially stained—that is, it will be found that certain tissues have taken up the colour, others remaining colourless. If now you put back the

animals into the tinted water and wait, you will find after a further lapse of time that further groups of tissues have become stained. Thus it was found by Ehrlich (*Biol. Centralb.*, vi, 1886 p. 214); *Abh. k. Akad. Wiss. Berlin*, February 25th, 1885) that on injection of the colour into living animals axis-cylinders of *sensory* nerves stain, whilst *motor* nerves remain colourless. [The motor nerves, however, will also stain, though later than the sensory nerves.] It might be supposed that by continuing the staining for a sufficient time, a point would be arrived at at which *all* the tissues would be found to be stained. This, however, is not the case. It is always found that the stained tissues only keep the colour that they have taken up for a short time after they have attained the maximum degree of coloration of which they are susceptible, and then begin to discharge the colour even more quickly than they took it up. According to Ehrlich this decoloration is explained as follows: methylen blue, on contact with reducing agents in alkaline solution, can be reduced to a colourless body, its "leuco-base." Living tissues readily reduce methylen blue. The leucobase thus formed is easily reoxidised into methylen blue by oxidising substances, or acids, or even by the mere contact of air—which latter property is taken advantage of in practice.

It follows that a total stain of all the tissues of a living intact organism can hardly be obtained under these conditions, but that a specific stain of one group or another of elements may be obtained in one or two ways. If the tissue to be studied be one that stains earlier than the others, it may be studied during life at the period at which it alone has attained the desired intensity of coloration. If it be one that stains later than the others, it may be studied at the period at which the earlier stained elements have already passed their point of maximum coloration and have become sufficiently decoloured, the later stained ones being at a point of desired intensity. Or the observer may fix the stain in either of these stages and preserve it for leisurely study by means of one of the processes given, § 382.

The proper strength of the very dilute solutions to be employed for the staining of living organisms must be made out by experiment for each object. We think the tint is practically a sufficient guide, but it may be stated that when in doubt a strength of 1 : 100,000 may be taken, and increased or diminished as occasion may seem to require. Zoja (*Rendic. R. Ist. Lombardo*, xxv, 1892; *Zeit. wiss. Mik.*, ix, 1892, p. 208) finds that for Hydra the right strength is from 1 : 20,000 to 1 : 10,000.

The stain is capricious. It is not possible to predict without trial which tissues will stain first in any organism. The stain penetrates very badly, which is no doubt one cause of its capriciousness. Gland cells generally stain early; then, in no definable order, other epithelium cells, fat cells, plasma cells, "Mastzellen," blood and lymph corpuscles, elastic fibres, smooth muscle, striated muscle. There are other elements that stain in the living state, but *not* when the staining is performed by

simple immersion of intact animals in a dilute staining solution in the manner we are considering. Chief amongst these are *nerve-fibres* and *ganglion-cells, which remain unstained in the intact organism.* To get these stained, it is necessary to isolate them sufficiently, as explained in the following sections.

379. Staining Nervous Tissue during Life. Ehrlich (*op. cit.*, last §) found that by injecting a solution of methylen blue into the vessels or tissues of living animals and shortly afterwards cutting out and examining small pieces of their tissues, these will be found to be intensely stained in some of their elements (chiefly nervous). If the tissues are mounted under a cover-glass, the stain will fade in a short time; but if the cover-glass be removed, so that oxygen can have access to the tissues, the stain will be restored, as explained last §. The chief elements stained in this way are peripheral nerves, and amongst these more especially axis-cylinders of sensory nerves.

Ehrlich held that the stain so obtained is a product of a *vital* reaction of the tissues, and that it cannot be obtained with dead material. Dogiel, however (*Arch. mik. Anat.*, xxxv, 1890, pp. 305 *et seq.*), found that muscle nerves of limbs of the frog could be stained as much as from three to eight days after the limbs had been removed from the animal. He concludes, indeed, that the reaction shows that the nerves were still living at that time. But it seems more natural to conclude with Apáthy (*Zeit. wiss. Mik.*, ix, 1892, pp. 15 *et seq.*) that nerve-tissue can be stained after life has ceased. Apáthy has directly experimented on this point, and sums up the necessary conditions as follows: The tissue need not be living, but must be fresh; nothing must have been extracted from it chemically, and its natural state must not have been essentially changed by physical means. For example the tissue must not have been treated with even dilute glycerin, nor with alcohol, though a treatment for a short time with physiological salt solution is not very hurtful; it must not have been coagulated by heat. Michailow (*ibid.*, xxvii, 1910, p. 7) prefers tissues that have lain from one and a half to two hours after the death of the subject in Ringer's salt solution.

As above explained, the primary stain obtained by injecting methylen blue, or immersing tissues in it, only lasts a very short time. In order to get it to last long enough for study, it must be re-blued by oxidation (see last §). It is therefore the usual practice to dissect out the tissues to be examined, and leave them for some time exposed to the air. This is done in order that they may take up from the air the necessary *oxygen*. Ehrlich also (*op. cit.*) holds that an alkaline reaction of the tissues is a necessary condition to the stain. Apáthy further holds that the stain is a *regressive* one, easily washed out by the surrounding liquid;

and in order to prevent this washing-out being excessively rapid, it is desirable to have it go on in presence of as little liquid as possible.

380. The Modes of Staining. The practice of the earlier workers at this subject was (following EHRLICH) to *inject* methylen blue into the vascular system or body-cavity of a living animal, wait a sufficient time, then remove the organ for further preparation and study. And there appears to have been a belief with some workers that it was essential that the stain should have been brought about by injection of the colouring matter into the *entire animal.* It is now known that the reaction can often be equally well obtained by removing an organ and subjecting it to a *bath* of the colouring matter in the usual way. But in some cases it seems that injection is preferable, if not necessary.

381. The Solutions Employed. The solutions used *for injection* generally made in salt solution (physiological, or a little weaker); those for staining by immersion, either in salt solution or other "indifferent" liquid, or in pure water. The earlier workers generally took concentrated solutions. Thus ARNSTEIN (*Anat. Anz.*, 1887, p. 125) injected 1 c.c. of saturated (*i.e.*, about 4 per cent.) solution into the vena cutanea magna of frogs and removed the organ to be investigated after the lapse of an hour. BIEDERMANN (*Sitzb. Akad. wiss. Wein. Math. Nat. Cl.*, 1888, p. 8) injected 0·5 to 1 c.c. of a nearly saturated solution in 0·6 per cent. salt solution into the thorax of cray-fishes and left the animals for from two to four hours before killing them. S. MAYER (*Zeit. wiss. Mik.*, vi, 1889, p. 423) took a strength of 1 : 300 or 400 of 0·5 per cent. salt solution. The solutions of RETZIUS are of the same strength. But the tendency of more recent practice is decidedly towards the employment of weaker solutions. APÁTHY (*ibid.*, ix, 1892, pp. 25, 26 *et seq.*) finds that it is not only superfluous, but positively disadvantageous, to take solutions stronger than 1 : 1000. DOGIEL (*Enzyk. Mik. Technik.*, 1st ed., p. 815) recommends $\frac{1}{8}$ to $\frac{1}{4}$ per cent. or at most $\frac{1}{2}$ per cent. For warm-blooded animals the solution should be warmed to 36° or 37° C., and before injecting the blood-vessels should be well washed out with similarly warmed salt solution. The injected organs may be removed after twenty to thirty minutes. They should be placed on a thin layer of spun glass moistened with weak ($\frac{1}{8}$ to $\frac{1}{15}$ per cent.) methylen blue, or simply spread out on a slide, and the whole placed in a Petri dish with a layer of the methylen blue on the bottom. The dish is best placed in a stove at 36° C., and after fifteen to thirty minutes (if the pieces are thin) or one hour to one and a half hours (if they are thick) specimens may be removed for examination or preservation; or, without using the stove, specimens may be removed from ten to twenty minutes after injection, placed on a slide, and moistened

with weak methylen blue or salt solution, and brought under the microscope. Then as soon as the stain is sufficiently brought out (from forty to sixty minutes) they may be fixed.

For staining *by immersion* the solutions should, if anything, be still weaker. Dogiel (*Arch. mik. Anat.*, xxxv, 1890, p. 305) places objects in a few drops of aqueous or vitreous humour, to which are added 2 or 3 drops of a $\frac{1}{16}$ to $\frac{1}{15}$ per cent. solution of methylen blue in physiological (0·75 per cent.) salt solution, and exposes them therein to the air. In thin pieces of tissues the stain begins to take effect in five or ten minutes, and attains its maximum in from fifteen to twenty minutes. For thicker specimens—retina, for instance—several hours may be necessary. The reaction is quickened by putting the preparations into a stove kept at 30° to 35° C. Rouget (*Compt. Rend.*, 1893, p. 802) employed a 0·05 per cent. solution in 0·6 per cent. salt solution (for muscles of Batrachia). Allen (*Quart. Jour. Micr. Sci.*, 1894, pp. 461, 483) takes for embryos of the lobster a solution of 0·1 per cent. in 0·75 per cent. salt solution, and dilutes it with 15 to 20 volumes of sea-water. Seidenmann (*Zeit. wiss. Mik.*, xvii, 1900, p. 239) takes for the choroid a solution of 0·02 per cent. in 0·5 per cent. salt solution. Lavdowsky (*ibid.*, xii, 1895, p. 177) takes $\frac{1}{10}$ to $\frac{1}{4}$ per cent. in white of egg, or serum. Similarly Young (*ibid.*, xv, 1898, p. 253). Michailow (*ibid.*, xxvii, 1910, p. 10) takes $\frac{1}{8}$ to $\frac{1}{32}$ per cent. in Ringer's salt solution (for nerves of mammals).

Apáthy (*Zeit. wiss. Mik.*, ix, 1892, p. 15; see also his *Mikrotechnik*, p. 172) proceeds as follows for *Hirudinea* and other invertebrates. A portion of the ventral cord is exposed, or dissected out. If it be desired to stain as many ganglion cells as possible, as well as fibres, the lateral nerves, as well as the connectives, should be cut through near a ganglion. The preparation is then treated with the stain. This is, for the demonstration chiefly of fibres in *Hirudo* and *Pontobdella*, either a 1 : 1000 solution in 0·5 to 0·75 per cent. salt solution, allowed to act for ten minutes; or a 1 : 10,000 solution allowed to act for an hour to an hour and a half; or a 1 : 100,000 solution allowed to act for three hours (*Lumbricus* requires twice these times; *Astacus* and *Unio* require three times; medullated nerves of vertebrates four times). For the demonstration of ganglion cells the stain is allowed to act three or four times as long.

The preparations from the 1 : 1000 solution are then washed in salt solution for an hour; those from the 1 : 10,000 solution for a quarter of an hour; those from the 1 : 100,000 solution need not be washed at all. They are then treated with one of the ammoniacal fixing and differentiating liquids described in § 382. This is done by pouring the liquid over them, and leaving them in it *without moving them about in it* for at least

an hour and by preference in the dark. The further treatment is described in § 382.

The object of the ammonia in these liquids is to *differentiate* the stain—to produce an artificial "secondary differentiation." It acts by washing out the absorbed colour from certain elements, others resisting longer.

See also, for Hirudinea, SÁNCHEZ, in *Trab. Lab. Invest. Boli. Univ. Madrid*, vii, 1909, fasc. 1—4, or *Zeit. wiss. Mik.*, xxvii, 1910, p. 393 (injection of solutions of 0·2, 0·1, or 0·05 per cent., with further treatment as Apáthy or Bethe).

382. Fixation of the Stain. The stain obtained by any of these methods may be fixed, and more or less permanent preparations be made by one or other of the following methods :

ARNSTEIN (*Anat. Anz.*, 1887, p. 551) puts the tissue for half an hour into saturated aqueous solution of picrate of ammonia.

S. MAYER (*Zeit. wiss. Mik.*, vi, 1889, p. 422) preferred a mixture of equal parts of glycerin and saturated picrate of ammonia solution, which served to fix the colour and mount the preparations in. This was also in principle the method of RETZIUS (*Intern. Monatsschr. Anat. Phys.*, vii, 1890, p. 328).

DOGIEL (*Enzyk. Mik. Techn.*, ii, p. 105) puts for from two to twenty-four hours into saturated aqueous picrate of ammonia, and then into equal parts of glycerin and the picrate solution. (Thin membranes, and the like, may be fixed with 1 or 2 per cent. of 2 per cent. osmic acid solution added to the picrate solution and stained with picro-carmine before putting into the glycerin mixture.)

Other workers have employed saturated solution of iodine in iodide of potassium (so ARNSTEIN) or picro-carmine (so FEIST, *Arch. Anat. Entwickel.*, 1890, p. 116; cf. *Zeit. wiss. Mik.*, vii, 1890, p. 231), the latter having the advantage of preserving the true blue of the stain if it be not allowed to act too long, and the preparation be mounted in pure glycerin.

Picric acid has been used by LAVDOWSKY, but this after careful study is rejected by DOGIEL.

APÁTHY (*op. cit.*, § 381) brings preparations either into a concentrated aqueous solution of picrate of ammonia *free from picric acid*, and containing 5 drops of concentrated *ammonia* for every 100 c.c.; or, which is generally preferable, into a 1 to 2 per cent. freshly prepared solution of neutral *carbonate of* ammonia saturated with picrate. They remain in either of these solutions, preferably in the dark, for *at least an hour*. They are then brought into a *small* quantity of saturated solution of picrate of ammonia in 50 per cent. glycerin, where they remain until thoroughly saturated. They are then removed into a saturated solution of the picrate in a mixture of 2 parts 50 per cent. glycerin, 1 part cold saturated sugar solution, and 1 part similarly prepared

gum-arabic solution. When thoroughly penetrated with this they are removed and mounted in the following gum-syrup medium (*loc. cit.*, p. 37):

Picked gum-arabic	50 grm.
Cane-sugar (not candied) . . .	50 ,,
Distilled water	50 ,,

Dissolve over a water-bath and add 0·05 grm. thymol. (This mounting medium sets quickly and as hard as balsam, so that no cementing of the mounts is necessary. Farrant's medium [with omission of the arsenious acid] will also do. In neither case should either ammonium picrate or methylen blue be added to the medium.) Preparations that have been *fully* differentiated (§ 381) do not keep more than a few weeks; whilst those in which the differentiation has not been carried to the point of thorough tinctorial isolation of the neuro-fibrils have kept for five or six years (APÁTHY, *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 712).

PLESCHKO (*Anat. Anz.*, xiii, 1897, p. 16) fixes with picrate, and then puts into 10 per cent. formol for a few days.

The methods described next § are also available for material not destined to be sectioned.

383. Methods for Sections. The preceding methods do not give preparations that will resist the operations necessary for imbedding in paraffin or mounting in balsam. A strong solution of platinum chloride is said to do this (see FEIST, *Arch. Anat. Entw.*, 1890, p. 116), but the preparations are not very satisfactory.

For the earlier method of PARKER (*Zool. Anzeig.*, 1892, p. 375) with methylal, see early editions. Later (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 4) he fixed the stain by dehydrating the objects in successive alcohols of 30, 50, 70, 95 and 100 per cent. strength, each containing 8 per cent. of corrosive sublimate, then brought them into a mixture of the last with an equal volume of xylol, and lastly into pure xylol.

For the earlier method of BETHE (*Arch. mik. Anat.*, xliv, 1894, p. 585), see last edition.

BETHE's later method (*Anat. Anz.*, xii, 1896, p. 438) is as follows: After staining, pieces of tissue of 2 to 3 mm. thickness are treated for ten to fifteen minutes with a concentrated aqueous solution of picrate of ammonia and then brought into a solution of 1 grm. of molybdate of ammonium, either in 20 of water, or in 10 of water and 10 of 0·5 per cent. osmic acid or 2 per cent. chromic acid; or into a solution of phosphomolybdate of sodium in the same proportions, each of these solutions having added to it 1 drop of hydrochloric acid, and if desired 1 grm. of peroxide of hydrogen. They remain in one of these solutions for three-quarters to one hour (or from four hours to twelve in the osmic acid one), and are then passed through water, alcohol, xylol, balsam, or paraffin.

(The objects that have been treated with one of the solutions of the sodium salt are not thoroughly resistant to alcohol, so that for them it is well to cool the alcohol to under 15° C.) Sections may be after-stained with alum carmine, or "neutral" tar colours.

Slight modifications of this method are given by DOGIEL (*Arch. mik. Anat.*, 1897, p. 772; 1898, p. 237; *Zeit. wiss. Zool.*, lxvi, 1899, p. 361; and *Enzyk. mik. Technik.*, 1903, p. 825, and 1910, p. 108). He omits the peroxide, the hydrochloric acid, and the cooling. BETHE (*Zeit. wiss. Mik.*, xvii, 1900, p. 21) does not approve of these modifications.

Further modifications of the molybdenum method have been published by LEONTOWITSCH (*Intern. Monatsschr. Anat.*, xviii, 1901, p. 142).

MICHAILOW (*Zeit. wiss. Mik.*, xxvii, 1910, p. 19) adds to 8 per cent. solution of molybdate 0·5 per cent. of formalin, leaves the objects in a large quantity of it (filtered) for twenty-four hours at 37° C., washes with warm water, and passes through alcohol and xylol into xylol-damar (not balsam).

See also SCHMIDT (*Arch. Ges. Phys.*, ciii, 1906, p. 522).

HARRIS (*Philadelphia Medical Journ.*, May 14th, 1898), after staining, rinses with water, and brings into a saturated solution of either ferrocyanide or ferricyanide of potassium which has been cooled to within a few degrees of zero (a trace of osmic acid may be added to prevent maceration). They remain therein for from three to twenty-four hours, and are then washed in distilled water for an hour, and are dehydrated in absolute alcohol kept at a low temperature, cleared in xylol or cedar oil, and imbedded in paraffin.

384. Impregnation of Epithelia, Lymph-spaces, etc. (DOGIEL, *Arch. mik. Anat.*, xxxiii, 1889, pp. 440 *et seq.*). Suitable pieces of tissue (thin membrane by preference) are brought fresh into a 4 per cent. solution of methylen blue in physiological salt solution (in the *Encycl. mik. Technik.*, 1903, p. 827, Dogiel gives the strength of the methylen blue as $\frac{1}{2}$ to 1 per cent.). After a few minutes therein they are brought into saturated solution of picrate of ammonia, soaked therein for half an hour or more, then washed in fresh picrate of ammonia solution, and examined in dilute glycerin.

If it be wished only to demonstrate the outlines of endothelium cells, the bath in the stain should be a short one, not longer than ten minutes in general; whilst if it be desired to obtain an impregnation of ground-substance of tissue, so as to have a negative image of other spaces, the staining should be prolonged to fifteen or thirty minutes.

If it be desired to preserve the preparations permanently, they had better be mounted in glycerin saturated with picrate of ammonia, or (*Enzyk.*, 1910, ii, p. 110) fixed with ammonium molybdate and a trace of osmium.

The effect is practically identical (except as regards the colour) with that of a *negative impregnation with silver nitrate.*

S. MAYER (*Zeit. wiss. Mik.*, vi, 1889, p. 422) stains tissues for about ten minutes in a 1 : 300 or 400 solution of methylen blue in 0·5 per cent. salt solution, rinses in salt solution, and puts up in the glycerin-picrate of ammonia mixture given § 382. The images are generally positive after injection of the colour into the vascular system; negative after immersion of the tissues.

TIMOFEJEW (*Anat. Anz.*, xxxv, 1909, p. 296) impregnates for fifteen to twenty minutes in a solution of 1 : 300 or 400 strength, fixes with a very weak solution of ammonium picrate in salt solution, and puts up in a mixture of 50 c.c. glycerin, 50 c.c. water, and 35 c.c. saturated solution of the picrate: or fixes with ammonium molybdate of 8 per cent. and mounts in balsam.

385. Toluidin Blue or Thionin as succedanea of methylen blue. HARRIS (*Philadelphia Med. Journ.*, May 14th, 1898) has found that there is no reaction of methylen blue that cannot be equally well obtained with toluidin blue or thionin. For staining pieces of tissue he takes:

Toluidin blue, 0·1 per cent. sol. in physiological salt solution	2 parts.
Ammonium chloride 0·25 per cent. in water	1 part.
Egg albumen	1 ,,

For injections he uses 1 part of the dye to 1000 of physiological salt solution.

Any of the methylen blue fixing methods may be employed and the whole technique is the same.

L. MARTINOTTI (*Zeit. wiss. Mik.*, xxvii, 1910, p. 24) recommends a *polychrome* toluidin blue, made by adding 0·5 per cent. of lithium carbonate to a 1 per cent. solution of the dye and keeping till a purple-red tone appears. Or, a stock solution made of 1 grm. toluidin blue, 0·5 grm. lithium carbonate, glycerin 20 c.c., alcohol 5 c.c., and water 75 c.c.

386. GOODPASTURE'S **Carbol-Fuchsin and Methylen Blue** (*Am. Jour. Path.*, i, 1925, 550). This method is very useful for the coloration of Negri bodies, bacteria, Rickettsiæ, and a wide variety of cytoplasmic inclusions. Sections from Zenker-fixed material are stained for ten minutes in:

Alcohol 20 per cent.	100·0 c.c.
Phenol (pure)	1·0 ,,
Anilin oil	1·0 ,,
Basic fuchsin	0·5 grm.

"The finely powdered or granular dye dissolves easily and the solution is immediately ready for use. Exposure to light and air causes a precipitation of the dye in about two months, so that the solution becomes weaker and must be discarded. It is convenient

to keep the required alcoholic solution of fuchsin, and to add the phenol and anilin oil when it is desired to make up the stain anew. If sections are stained in the solution for an hour or more they gradually become useless. In our experience it is safe to stain the sections as long as half an hour, but the best results are obtained in ten minutes.

"Wash off the excess stain in running water, blot with filter paper, and decolorise in 95 per cent. alcohol until the sections become pink. Wash off in water and counterstain 15 to 60 seconds with Loeffler's methylen blue. Wash in water. Dehydrate and decolorise for a few seconds in absolute alcohol (until the excess blue is removed). Clear in xylol; mount in balsam."

CHAPTER XIX

METALLIC STAINS (IMPREGNATION METHODS)

387. The Characters of Impregnation Stains. By impregnation is understood a mode of coloration in which a metal is deposited in tissues in the form of a *precipitate*—the impregnated elements becoming in consequence opaque. By staining, on the other hand, is understood a mode of coloration in which the colouring matter is retained by the tissues as if in a state of *solution*, showing no visible solid particles under the microscope, the stained elements remaining in consequence transparent. But it is not right to draw a hard and fast line between the two kinds of coloration. Some of the metallic salts treated of in this chapter give, besides an impregnation, in some cases a true stain. And some of the dyes that have been treated of in the preceding chapters give, besides a stain, a true impregnation. Methylen blue, for instance, will give in one and the same preparation an impregnation and a stain; and in most chloride preparations the coloration is in places of the nature of a finely divided solid deposit, in others a perfectly transparent stain.

Negative and Positive Impregnations. In a *negative* impregnation intercellular substances alone are coloured, the cells themselves remaining colourless or very lightly tinted. In a *positive* impregnation the cells are stained and the intercellular spaces are unstained.

Negative impregnation is generally held to be *primary* because brought about by the direct reduction of a metal in the intercellular spaces; positive impregnation to be *secondary* (in the case of silver nitrate at least) because it is brought about by the solution in the liquids of the tissues of the metallic deposit formed by a primary impregnation, and the consequent staining of the cells by the new solution of metallic salt thus formed. These secondary impregnations take place when the reduction of the metal in the primary impregnation is not sufficiently energetic (see on these points HIS, *Schweizer Zeit. Heilk.*, ii, Heft 1, p. 1; GIERKE, *Zeit. wiss. Mik.*, i, p. 393; RANVIER, *Traité*, p. 107).

As to the nature of the black or brown deposit or stain formed in the intercellular spaces in cases of primary impregnation see SCHWALBE, *Arch. mik. Anat.*, vi, 1870, p. 5; GIERKE'S *Farberei zu mikroskopischen Zwecken*, in vols. i and ii of *Zeit. wiss. Mik.*; JOSEPH, *Sitzb. Akad. Wiss. Berlin*, 1888; *Zeit. wiss. Mik.*, xi, 1, 1894, pp. 42 *et seq.* It evidently cannot consist of metallic silver, as it is soluble in hyposulphite of soda. See also MACALLUM, *Proc. Roy. Soc.*, lxxvi, 1905, p. 217, and ACHARD and REYNAUD, *C.R. Soc. Biol.*, lxi, 1906, p. 43.

388. Action of Light on Solutions of Metallic Salts. Stock solutions of metallic salts are generally kept in the dark, or at least in coloured bottles, under the belief that exposure to light reduces them. It has been pointed out in § 40 that in the case of osmic acid, not light, but *dust* is the reducing agent, and that solutions may be exposed to light with impunity if dust be absolutely denied access to them. We have now good evidence to the effect that the same is the case with other metallic solutions ; and the point is raised whether such solutions are not positively improved for impregnation purposes by exposure to light ! Dr. Lindsay Johnson wrote to Lee as follows :

" One may (I find by experiment) state as a rule without exception that all the solutions of the chlorides and nitrates of the metals will keep indefinitely in clean white stoppered bottles in the sunlight ; and as far as osmium, uranium, gold and silver, and platinum are concerned, actually improve or ripen by a good sunning. All photographers tell me their papers salt more evenly by old well-sunned silver nitrate than by a fresh solution kept in the dark ; and I go so far as to say that this is one of the reasons why gold stains are so unsatisfactory."

Apáthy (*Mitt. Zool. Stat. Neapel*, xii, 1897, p. 722) leaves his gold solutions exposed to light, so long as there are no tissues in them.

389. State of the Tissues to be Impregnated. The majority of stains given by *dyes* are only obtained with tissues that have been changed in their composition by the action of fixing and preservative reagents. With metallic impregnations the case is different ; perfectly *fresh* tissues—that is, such as are either living, or at all events have not been treated by any reagent whatever—will also impregnate with the greatest ease and precision. Indeed, some impregnations will not succeed at all with tissues that are not fresh in the sense above explained.

SILVER

390. Silver Nitrate : Generalities. The principles of its employment are given by Ranvier (*Traité*, p. 105) as follows :

Silver nitrate may be employed either in solution or in the solid state. The latter method is useful for the study of the cornea and of fibrous tissues, but is not suitable for epithelia. For the cornea, for instance, proceed as follows : The eye having been removed, a piece of silver nitrate is quickly rubbed over the anterior surface of the cornea, which is then detached and placed in distilled water ; it is then brushed with a camel's hair brush in order to remove the epithelium. The cornea is then exposed to the action of light. It will be found that the nitrate has traversed the epithelium and soaked into the fibrous tissue, on the surface of which it is reduced by the light. The cells of the tissues will be found unstained.

It is generally employed in solution, in the following manner : In the case of a membrane, such as the epiploön, the membrane

must be *stretched* like a drum-head over a porcelain dish, and *washed* first with distilled water, and then washed with a solution of silver nitrate. In order to obtain a powerful stain it is necessary that this part of the operation be performed in direct sunlight, or at least in a very brilliant light. As soon as the tissue has begun to turn a blackish grey the membrane is removed, washed in distilled water, and *mounted* on a slide in some suitable examination medium.

If the membrane were left in the water the cells would become detached, and would not be found in the finished preparation.

If the membrane had not been stretched as directed the silver would be precipitated not only in the intercellular spaces, but in all the small folds of the surface.

If the membrane had not been washed with distilled water before impregnation there would have been formed a deposit of silver on every spot on which a portion of an albuminate was present, and these deposits might easily be mistaken for a normal structure of the tissue. It is thus that impurities in the specimen have been described as stomata of the tissue.

If the solution be taken too weak—for instance, 1 : 500 or 1 : 1000, or if the light be not brilliant—a *general* instead of an *interstitial* stain will result; nuclei will be most stained, then protoplasm, and the intercellular substance will contain but very little silver. In general in a good "impregnation" the contents of the cells, and especially nuclei, are quite invisible.

The tissues should be constantly *agitated* in the silver-bath in order to avoid the formation on their surfaces of deposits of chlorides and albuminates of silver.

These impregnations only succeed with *fresh* tissues.

391. Silver Nitrate: the Solutions to be employed (Ranvier). The solutions generally employed by Ranvier vary in strength from 1 : 300 to 1 : 500. Thus 1 : 300 is used for the epiploön, pulmonary endothelium, cartilage, tendon; whilst a strength of 1 : 500 is employed for the phrenic centre, and the epithelium of the intestine. For the endothelium of blood-vessels (by injection) solutions of 1 : 500 to 1 : 800 are taken.

M. Duval (*Précis*, p. 229) takes solutions of 1, 2, or at most 3 per cent.

v. Recklinghausen used, for the cornea, a strength of from 1 : 400 to 1 : 500 (*Die Lymphgefässe*, etc., Berlin, 1862, p. 5).

Robinski (*Arch. de Physiol.*, 1869, p. 451) used solutions varying between 0·1 and 0·2 per cent., which he allowed to act for thirty seconds.

Rouget (*Arch. de Physiol.*, 1873, p. 603) employed solutions as weak as 1 : 750, or even 1 : 1000, exposing the tissues to their action several times over, and washing them with water after each bath.

The HERTWIGS take, for marine animals, a 1 per cent. solution (*Jen. Zeit. Naturk.*, xvi, pp. 313 and 324).

The HOGGANS (*Journ. of Anat. and Physiol.*, xv, 1881, p. 477) take for lymphatics a 1 per cent. solution.

TOURNEUX and HERRMANN (ROBIN'S *Journal de l'Anat.*, 1876, p. 200) took for the epithelia of Invertebrates 3 : 1000, and in some cases weaker solutions—for one hour, washing out with alcohol of 90 per cent.

HOYER (*Arch. mik. Anat.*, 1867, p. 649) takes a solution of nitrate of silver, and adds ammonia to it until the precipitate that is formed just redissolves, then dilutes the solution until it contains from 0·75 to 0·50 per cent. of the salt. This *ammonio-nitrate* solution has the advantage of impregnating absolutely nothing but endothelium or epithelium; connective tissue is not affected by it.

RANVIER'S *injection-mass* for impregnating endothelium is given under "Injection."

DEKHUYZEN (*Anat. Anz.*, iv, 1889, No. 25, p. 789) has applied to terrestrial animals the method of HARMER for marine animals (§ 395). For details see *previous editions.*

REGAUD (*Journ. Anat. et Phys.*, xxx, 1894, p. 719) recommends for the study of lymphatics a process devised by RENAUT, for the details of which see also *previous editions.*

392. Other Salts of Silver. ALFEROW (*Arch. Phys.*, i, 1874, p. 694) employs the picrate, lactate, acetate, and citrate, in solution of 1 : 800, and adds a small quantity of the acid of the salt taken (10 to 15 drops of a concentrated solution of the acid to 800 c.c. of the solution of the salt). This decomposes the precipitates formed by the action of the silver salt on the chlorides, carbonates, and other substances existing in the tissues.

REGAUD and DUBREUIL (*C.R. Ass. Anat.*, 5 Sess. 1903, p. 122) take a fresh solution of protargol or a mixture of equal parts of 1 per cent. protargol and 1 per cent. osmic acid, avoiding precipitates.

393. Silver Nitrate : Reduction. Reduction may be effected in media other than distilled water.

v. RECKLINGHAUSEN washed his preparations in salt solution before exposing them to the light in distilled water (*Arch. path. Anat.*, xix, p. 451). Physiological salt solution (0·75 per cent.) is commonly used for these washings.

MÜLLER (*Arch. f. path. Anat.*, xxxi, p. 110), after impregnation by immersion for two or three minutes in a 1 per cent. solution of nitrate of silver in the dark, adds to the solution a small quantity of 1 per cent. solution of iodide of silver (dissolved by the aid of a little iodide of potassium). After being agitated in this mixture the preparations are washed with distilled water, and exposed to the light for two days in a 1 per cent. solution of nitrate of silver (see also GIERKE, in *Zeit. wiss. Mik.*, i, 1884, p. 396).

ROUGET (*Arch. de Physiol.*, 1873, p. 603) reduces in glycerin;

Szutz (*Zeit. wiss. Mik.*, xxix, 1912, p. 291) in glycerin with $\frac{1}{10}$ of formol.

Sattler (*Arch. mik. Anat.*, xxi, p. 672) exposes to the light for a few minutes in water acidulated with acetic or formic acid.

Thanhoffer (*Das Mikroskop*, 1880) employs a 2 per cent. solution of acetic acid.

Krauss brings his preparations, after washing, into a light red solution of permanganate of potash. Reduction takes place very quickly, even in the dark.

Oppitz puts for two or three minutes into a 0·25 or 0·50 per cent. solution of chloride of tin.

Jakimovitch (*Journ. de l'Anat.*, xxiii, 1888, p. 142) brings nerve preparations, as soon as they have become of a dark brown colour, into a mixture of formic acid 1 part, amyl alcohol 1 part, and water 100 parts, and exposes to the light for five to seven days, the mixture being renewed from time to time.

Dekhuyzen (*op. cit.*, last §) reduces in oil of cloves, after dehydration.

394. **Fixation.** Legros (*Journ. de l'Anat.*, 1868, p. 275) washes his preparations, after reduction, in hyposulphite of soda, to prevent after-blackening. According to Duval (*Précis*, p. 230) they should be washed for a few seconds only in 2 per cent. solution and then in distilled water.

Gerota (*Arch. Anat. Phys., Phys. Abth.*, 1897, p. 428) reduces in a hydroquinone developing solution, followed by fixation in hyposulphite of soda, just as in photography.

395. **Impregnation of Marine Animals.** On account of the chlorides that bathe the tissues of marine animals, these cannot be treated *directly* with nitrate of silver.

Hertwig (*Jen. Zeit.*, xiv, 1880, p. 322) recommends fixing them with a weak solution of osmic acid, then washing with distilled water until the wash-water gives no more than an insignificant precipitate with silver nitrate, and then treating for six minutes with 1 per cent. solution of silver nitrate.

Harmer (*Mitth. Zool. Stat. Neapel*, v, 1884, p. 445) washes them for some time (half an hour) in a 5 per cent. solution of nitrate of potash in distilled water; they may then be treated with silver nitrate in the usual way. For some animals he recommends a 4·5 per cent. solution of sulphate of soda.

396. **Double-staining Silver-stained Tissues.** The nuclei of tissues impregnated with silver may be stained with the usual reagents, provided that solutions containing free ammonia be avoided. These stains will only succeed, however, with successful negative impregnations, as nuclei that have been impregnated will not take the second stain.

Impregnation with silver may be followed by impregnation with gold. In this case the gold generally substitutes itself for the silver in the tissues, and though the results are sharp and

precise, the effect of a double stain is not produced. See hereon GEROTA, *loc. cit.*, § 394.

397. Impregnation of Nerve Tissue. *For this subject, which includes the important bichromate-and-silver method of* GOLGI, *and the neurofibril methods of* BIELSCHOWSKY *and* RAMÓN Y CAJAL, *see Part II. These give important results, not only with Nervous tissue, but with various forms of Connective tissue, mitochondrial formations, etc.*

GOLD

398. The Characters of Gold Impregnations. Gold chloride differs from nitrate of silver in that it generally gives *positive* (§ 387) impregnations only. It generally gives negative images only with such tissues as have first received a negative impregnation with silver, the gold substituting itself for the silver. In order to obtain these images you first impregnate very lightly with silver; reduce; treat for a few minutes with a 0·5 per cent. solution of gold chloride, and reduce in acidulated distilled water.

This process, however, is in but little use, and except for certain special studies on the cornea and on connective tissue, the almost exclusive function of gold chloride is the impregnation of nervous tissue, for which it exhibits a remarkable selectivity.

399. Pre-impregnation and Post-impregnation. Gold methods may be divided into two groups: viz. *pre-impregnation* methods, characterised by employing *perfectly fresh* tissues, and *post-impregnation* methods, characterised by the employment of *fixed and hardened* tissues. Both are chiefly used for *nervous* tissue. They give in some respects opposite results. Pre-impregnation gives nuclei unstained, cytoplasm rather strongly stained, axis-cylinders reddish-violet. Post-impregnation gives nuclei sharply stained, cytoplasm pale, axis-cylinders black, and (when successful) showing their neurofibrils sharply distinguished from the interfibrillar substance.

In APÁTHY's view (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 718) successful gold preparations should show a true *stain*, not an impregnation (§ 387), the stain being brought about by the formation of gold oxide (AuO) which combines with the tissue elements. He advises in consequence that preparations should *not be moved about* more than can be helped in the reducing bath, so that the colouring oxide may not be washed away from the tissues before the stain has taken effect.

400. As to the Commercial Salts of Gold. SQUIRE's *Methods and Formulæ*, etc. (p. 43), says: " Commercial chloride of gold is not the pure chloride, $AuCl_3$, but the crystallised double chloride of gold and sodium, containing 50 per cent. of metallic gold.

" Commercial chloride of gold and sodium is the above crystal-

lised double chloride mixed with an equal weight of chloride of sodium, and contains 25 per cent. of metallic gold."

This, however, appears not to be the case in Germany. Dr. GRÜBLER, writing to MAYER (see the *Grundzüge*, LEE und MAYER, p. 215), says: "*Aurum chloratum fuscum* contains about 53 per cent. Au, the *flavum* about 48 per cent.; in both of them there should be only water and hydrochloric acid besides the gold, no sodium chloride. Pure *Auronatrium chloratum* contains 14·7 per cent. of sodium chloride, though samples are found in commerce with much more."

APÁTHY (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 722) formerly employed the *aurum chloratum flavum*, but now prefers the *fuscum*.

A. PRE-IMPREGNATION

401. The State of the Tissues to be Impregnated. The once classical rule, that for researches on nerve-endings the tissues should be taken perfectly fresh, seems not to be valid for all cases. For DRASCH (*Sitzb. Akad. wiss. Wien*, 1881, p. 171, and 1884, p. 516; and *Abhand. math.-phys. Cl. K. Sach. Ges. Wiss.*, xiv, No. 5, 1887; *Zeit. wiss. Mik.*, iv, 1887, p. 492) finds that better results are obtained with tissues that have been allowed to lie after death for twelve, twenty-four, or even forty-eight hours in a cool place.

402. COHNHEIM'S **Method** (*Virchow's Arch.*, Bd. xxxviii, pp. 346—349; *Stricker's Handb.*, p. 1100). Fresh pieces of cornea (or other tissue) are put into 0·5 per cent. solution of chloride of gold until thoroughly yellow, and then exposed to the light in water acidulated with acetic acid until the gold is thoroughly reduced, which happens in the course of a few days at latest. They are then mounted in acidulated glycerin. Results very uncertain and anything but permanent.

403. LÖWIT'S **Method** (*Sitzgsber. Akad. Wien*, Bd. lxxi, 1875, p. 1). The following directions are from FISCHER'S paper on the corpuscles of Meissner (*Arch. mik. Anat.*, xii, 1875, p. 366).

Small pieces of *fresh* skin are put into *dilute formic acid* (1 volume of water to 1 of the acid of 1·12 sp. gr.), and remain there until the epidermis peels off. They then are put for fifteen minutes into gold chloride solution ($1\frac{1}{2}$ to 1 per cent.), then for twenty-four hours into dilute formic acid (1 part of the acid to 1—3 of water), and then for twenty-four hours into undiluted formic acid. (Both of these stages are gone through in the dark.) Sections are then made and mounted in damar or glycerin. Successful preparations show the nerves alone stained.

404. RANVIER'S **Formic Acid Method** (*Quart. Journ. Mic. Sci.* [N.S.], lxxx, 1880, p. 456). The tissues are placed in a *mixture of chloride of gold and formic acid* (4 parts of 1 per cent. gold chloride to 1 part of formic acid) which has been boiled and

allowed to cool (RANVIER'S *Traité*, p. 826). They remain in this until thoroughly impregnated (muscle twenty minutes, epidermis two to four hours); reduction is affected either by daylight in acidulated water, or in the dark in dilute formic acid (1 part of the acid to 4 parts of water).

405. RANVIER'S **Lemon-juice Method** (*Traité*, p. 813). RANVIER finds that of all acids lemon juice is the least hurtful to nerve-endings. He therefore soaks pieces of tissue in fresh lemon juice until they become transparent (five or ten minutes in the case of muscle). They are then rapidly washed in water, brought for about twenty minutes into 1 per cent. gold chloride solution, washed again in water, and brought into a mixture of 50 c.c. of distilled water and 2 drops of acetic acid. They are exposed to the light for twenty-four to forty-eight hours. The preparations thus obtained are good for immediate study, but are not permanent, the reduction of the gold being incomplete. In order to obtain perfectly reduced, and therefore permanent, preparations, the reduction should be done in the dark in a few cubic centimetres of dilute formic acid (1 part acid to 4 of water), which takes about twenty-four hours.

406. VIALLANE'S **Osmic Acid Method** (*Hist. et Dév. des Inseects*, 1883, p. 42). The tissues are treated with osmic acid (1 per cent. solution) until they begin to turn brown, then with 25 per cent. formic acid for ten minutes; they are then put into solution of chloride of gold of 1 : 5000 (or even much weaker) for twenty-four hours in the dark, then reduced in the light in 25 per cent. formic acid. Lee found this an excellent method.

KERSCHNER (*Arch. mik. Anat.*, lxxi, 1908, p. 522) puts till brown into a mixture of 10 parts 5 per cent. formic acid with 1 part 2 per cent. osmic acid, washes, puts for two to six hours into 1 per cent. gold chloride in the dark, washes, puts for twelve hours into 25 per cent. formic acid in the dark and then for twenty-four in the light, and mounts in 50 per cent. glycerin with 1 per cent. of formol.

407. Other Methods. The numerous other methods that have been proposed differ from the foregoing partly in respect of the solutions used for impregnation, but chiefly in respect of details imagined for the purpose of *facilitating the reduction* of the gold.

Thus BASTIAN employed a solution of gold chloride of a strength of 1 to 2000, acidulated with HCl (1 drop to 75 c.c.), and reduced in a mixture of equal parts of formic acid and water *kept warm*.

HÉNOCQUE (*Arch. de l'Anat. et de la Physiol.*, 1870, p. 111) impregnates in a 0·5 per cent. solution of gold chloride, washes in water for twelve to twenty-four hours, and reduces in a nearly saturated solution of *tartaric acid* at a temperature of 40° to 50° C. Reduction is effected very rapidly, sometimes in a quarter of an hour.

Hoyer (*Arch. mik. Anat.*, ix, 1873, p. 222) says that the double chloride of *gold and potassium* has many advantages over the simple gold chloride. He impregnates in solutions of 0·5 per cent. strength, and reduces in water containing 1 or 2 drops of a *pyrogallic acid* developing solution, such as is used in photography, or in a warm concentrated solution of tartaric acid, at the temperature of an incubating stove.

Lee used the double chloride of *gold and sodium* with good results.

Ciaccio (*Journ. de Microgr.*, vii, 1883, p. 38) prefers the double chloride of *gold and cadmium.*

Flechsig (*Die Leitungsbahnen in Gehirn*, 1876; *Arch. Anat. u. Phys.*, 1884, p. 453) reduces in a 10 per cent. solution of *caustic soda.*

Nesteroffsky treats impregnated preparations with a drop of *ammonium sulphide*, and finishes the reduction in glycerin (quoted from Gierke's *Färberei z. mik. Zwecken*).

Bohm reduces in *Pritchard's solution*—amyl alcohol, 1; formic acid, 1; water, 98.

Manfredi (*Arch. per le Sci. med.*, v, No. 15) puts fresh tissues into gold chloride, 1 per cent., for half an hour; then *oxalic acid*, 0·5 per cent., in which they are warmed in a water-bath to 36°. Mount in glycerin. Sunny weather is necessary.

Boccardi (*Lavori Instit. Fisiol. Napoli*, 1886, i, p. 27; *Journ. Roy. Mic. Soc.*, 1888, p. 155) recommends oxalic acid of 0·1 per cent. or of 0·25 to 0·3 per cent., or a mixture of 5 c.c. pure formic acid, 1 c.c. of 1 per cent. oxalic acid, and 25 c.c. of water, reducing in the dark not longer than two to four hours.

Kolossow (*Zeit. wiss. Mik.*, v, 1888, p. 52) impregnates for two or three hours in a 1 per cent. solution of gold chloride *acidulated* with 1 per cent. of HCl, and reduces for two or three days in the dark in a 0·01 per cent. to 0·02 per cent. solution of *chromic acid.*

Geberg (*Intern. Monatsschr.*, x, 1893, p. 205) states that previous treatment of tissues for twenty-four hours with *lime-water* (Arnstein's method) greatly helps the reduction.

Bernheim (*Arch. Anat. Phys., Phys. Abth.*, 1892, Supp., p. 29) adds to Löwit's dilute formic acid a piece of *sulphite of sodium* (must be fresh and smell strongly of sulphurous acid).

Dr. Lindsay Johnson writes that besides the "sunning" of the impregnating solution recommended above (§ 356), the gold should be carefully *acidulated with a neutral acetate* or formate, or acetic or formic acid, at least twenty-four hours before using; and then afterwards the tissue must be washed until no reaction occurs to test-paper.

Apáthy (*Mikrotechnik*, p. 173; *Mitth. Zool. Stat. Neapel*, xii, 1897, pp. 718—728) lays stress on the necessity of having the

objects *thoroughly penetrated by light* from all sides during the process of reduction. Objects, therefore, should always be so thin that light can readily stream through them. He impregnates for a few hours in 1 per cent. gold chloride (§ 400) in the dark, then brings the objects *without washing out with water*, the gold solution being just superficially mopped up with blotting-paper, into 1 per cent. formic acid. They are to be set up in this, in a tube or otherwise, so that the light *may come through them from all sides*, and exposed to diffused daylight in summer, or direct sunlight in winter, for six to eight hours *without a break*. They must *not be moved about* more than can be helped in the acid. If the acid becomes brown it may be changed for fresh. The temperature of the acid should not be allowed to rise over 20° C., whence direct sunlight is to be avoided during the summer. He mounts in glycerin or his syrup (§ 382). He finds such preparations *absolutely permanent*.

POST-IMPREGNATION

408. GERLACH'S **Method** (STRICKER'S *Handb.*, 1872, p. 678): Spinal cord is hardened for fifteen to twenty days in a 1 to 2 per cent. solution of bichromate of ammonia. Thin sections are made and thrown into a solution of 1 part of double chloride of gold and potassium to 10,000 parts water, which is very slightly acidulated with HCl, and after ten to twelve hours are washed in hydrochloric acid of 1 to 2 : 3000 strength, then brought for ten minutes into a mixture of 1 part HCl to 1000 parts of 60 per cent. alcohol, then dehydrated and mounted in balsam.

See further, for Nerve Centres, under "Nervous System."

409. GOLGI (*Mem. Accad. Torino* [2], xxxii, 1880, p. 382) puts tissues previously hardened in 2 per cent. solution of bichromate of potash for ten to twenty minutes into 1 per cent. solution of arsenic acid, then into $\frac{1}{2}$ per cent. solution of chloride of gold and potassium for half an hour, washes in water, and reduces in sunlight in 1 per cent. arsenic acid solution, which is changed for fresh as fast as it becomes brown. Mount in glycerin. Sunny weather is necessary.

410. APÁTHY'S **Method** (*Zeit. wiss. Mik.*, x, 1893, p. 349; *Mitth. Zool. Stat. Neapel*, xii, 1897, p. 729): The material to be used must have been fixed either in sublimate or in a mixture of equal parts of saturated solution of sublimate in 0·5 per cent. salt solution and 1 per cent. osmic acid (this more particularly for Vertebrates). The material should be imbedded *as quickly as possible*, either in paraffin or in celloidin. Sections are made and fixed on slides, and after the usual treatment with iodine, etc., are either put into distilled water for from two to six hours, or are rinsed in water, treated for one minute with 1 per cent. formic acid, and again well washed with water.

They are then put for twenty-four hours, or at least overnight, into the gold-bath, which is preferably 1 per cent. gold chloride (see § 400), but may be weaker, down to 0·1 per cent., after which they are just rinsed with water or superficially dried with blotting-paper. The slides are then set up on end in a sloping position, the sections looking downwards, so that precipitates may not fall on them, in glass tubes filled with 1 per cent. formic acid. The tubes are then exposed to light until the gold is reduced, *as directed in* § 407 *sub fin.*

Lee found it advantageous to reduce in weak solution of *formaldehyde*, either with or without formic acid.

Szütz (*Zeit. wiss. Mik.*, xxix., 1912, p. 292) reduces as Apáthy for one day, then rinses and puts back for the night into the gold, then for the next day again into the formic acid.

411. Impregnation of Marine Animals. For some reason the tissues of marine animals do not readily impregnate with gold in the fresh state. It is said by Fol that impregnation succeeds better with spirit specimens.

412. Preservation of Impregnated Preparations. Preparations may be mounted either in balsam or in acidulated glycerin (1 per cent. formic acid).

Theoretically they ought to be permanent if the reduction of the metal has been completely effected, but they are very liable to go wrong through after-blackening. Ranvier states that this can be avoided by putting them for a few days into alcohol, which he says possesses the property of stopping the reduction of the gold.

Blackened preparations may be *bleached* with cyanide or ferricyanide of potassium. Redding employs a weak solution of ferricyanide, Cybulsky a 0·5 per cent. solution of cyanide.

Preparations may be double-stained with the usual stains (safranin being very much to be recommended), but nuclei will only take the second stain in the case of negative impregnation.

OTHER METALLIC STAINS

413. Osmic Acid and Pyrogallol. This method was first published by Lee in 1887 (*La Cellule*, iv, p. 110). It consists in putting tissues that have been treated with osmic acid into a weak solution of pyrogallol, in which they quickly turn greenish-black, sometimes too much so.

Hermann (*Arch. mik. Anat.*, xxxvii, 4, 1891, p. 570) put platino-aceto-osmic material hardened in alcohol for twelve to eighteen hours into raw *pyroligneous* acid. This acid ought (*Ergebnisse der Anat.*, ii, 1893, p. 28) to be as raw as possible, and to be of a dark brown colour and evil-smelling. (The stain obtained in this way is *not* due to a mere reduction of the osmic acid, but

also to coloration by the brown pyroligneous acid; for HERMANN has obtained the same stain with sublimate material, or alcohol material (*op. cit.*, i, 1891 [1892], p. 7).)

Lee found this gives much better results than the pure osmic acid process, but not the best possible. Lee proceeded as follows:

HERMANN or FLEMMING material is brought *in bulk, directly after fixing,* into a weak aqueous solution of *pyrogallol.* The tissues may remain in it for twenty-four hours, but for small objects an hour or less is sufficient. An *alcoholic* solution of pyrogallol *may* be taken if desired. RAWITZ (*Lehrbuch*, p. 60) takes 20 per cent. aqueous sol. of tannin.

There is thus obtained a black stain, which is at the same time a plasma stain and a nuclear stain, chromatin being so far stained that it is not necessary to have recourse afterwards to a special chromatin stain. With Invertebrates it sometimes gives very elegant differentiations of nervous tissue. It is a *very easy* method, and if pyrogallol be used a very *safe* one (with pyroligneous acid not so safe).

If it be desired to add a chromatin stain, Lee recommended safranin (stain very strongly, twenty-four hours at least, and start the extraction with acid alcohol).

This method has been attributed to VON MAEHRENTHAL. See also under "Nervous System" modifications of this method by AZOULAY and HELLER and GUMPERTZ; also one by KOLOSSOW (*Zeit. wiss. Mik.*, ix, 1892, p. 38, and ix, 1893, p. 316).

414. Perchloride of Iron. This reagent, introduced by POLAILLON (*Journ. de l'Anat.*, iii, 1866, p. 43), sometimes gives useful results, especially in the study of peripheral nerve-ganglia, in which it stains the nervous tissue alone, the connective tissue remaining colourless.

The HOGGANS proceed as follows (*Journ. Quekett Club*, 1876; *Journ. Roy. Mic. Soc.*, ii, 1879, p. 358): The tissue (having been first fixed with silver nitrate, which is somewhat reduced by a short exposure to diffused light) is dehydrated in alcohol, and treated for a few minutes with 2 per cent. solution of perchloride of iron in spirit; then with a 2 per cent. solution of pyrogallic acid in spirit, and in a few minutes more, according to the depth of tint required, may be washed in water and mounted in glycerin.

FOL fixes in perchloride (§ 85) and treats for twenty-four hours with alcohol containing a trace of gallic acid.

POLAILLON (*loc. cit.*) reduces in tannic acid.

The method is not applicable to chromic objects.

GOLODETZ and UNNA (*Monats. prakt. Derm.*, xlviii, 1909, p. 153). put sections of skin for five minutes into fresh mixture of 1 per cent. perchloride of iron and 1 per cent. sol. of ferricyanide of potassium. See also UNNA and GOLODETZ, *ibid.*, xlix, 1909, p. 97.

ROOSEVELT (*Med. Rec.*, ii, 1887, p. 84; *Journ. Roy. Mic. Soc.*, 1888, p. 157) employs a stain composed of 20 drops of saturated solution of iron sulphate, 30 grm. water, and 15 to 20 drops pyrogallic acid.

415. Palladium Chloride (see SCHULZE, § 82). **Prussian Blue** (see LEBER, *Arch. Ophthalm.*, xiv, p. 300; RANVIER, *Traité*, p. 108). **Cupric**

Sulphate (see LEBER, *ibid.*). **Lead Chromate** (see LEBER, *ibid.*). **Sulphides** (see LANDOIS, *Centralb. med. Wiss.*, 1885, No. 55 ; and GIERKE, in *Zeit. wiss. Mik.*, i, 1884, p. 497). **Molybdate of Ammonia** (MERKEL ; KRAUSE) (see GIERKE, *ibid.*, i, 1884, p. 96). **Oxychloride of Ruthenium** (NICOLLE and CANTACUZÈNE) (see *Ann. Inst. Pasteur*, vii, 1893, p. 331). **Ruthenium Red** (Ruthenium Sesquichloride) (EISEN, *Zeit. wiss. Mik.*, xiv, 1897, p. 200 ; in our hands totally useless). **Oxide of Manganese** (GOLODETZ and UNNA, *Monats. prakt. Derm.*, xlviii, 1909, p. 151).

CHAPTER XX

OTHER STAINS AND COMBINATIONS

416. Kernschwarz (PLATNER, *Zeit. wiss. Mik.*, iv, 1887, p. 350). A black liquid on sale by Grübler. MAYER (*Grundzüge*, LEE & MAYER, 1st ed., p. 202) finds that it contains iron, combined with some gallic acid. Lee used it as follows :

Sections are fixed on slides and treated with Kernschwarz until the required depth of stain is obtained, which will be from a few minutes to twenty-four hours, according to the material.

There is obtained a black or neutral tint stain, which is either a pure chromatin stain, or at the same time a plasma stain. If overstaining should have occurred, the stain is easily differentiated by means of any weak acid, either in water or alcohol. PLATNER took alkalies, preferably carbonate of lithia, for differentiation.

It may be well, if a good plasma stain has been obtained, to afterstain for twenty-four hours with safranin, followed by differentiation in either neutral or acid alcohol, and clove oil. The stain is perfectly permanent in balsam, and is stated to be a good one for preparations that it is desired to photograph.

Lee recommends this stain which is safe and easy. The combination with safranin gives a better chromatin stain than safranin alone.

417. Brazilin, the colouring matter of Brazilian redwood or Pernambuco wood, has been recommended by EISEN (*Zeit. wiss. Mik.*, xiv, 1897, p. 198) and HICKSON (*Nature*, lxii, 1900, p. 589, and *Quart. Journ. Mic. Sci.*, 1901, p. 469). MAYER (*Grundzüge*, p. 203) finds that, in alum solution, it gives a stain similar to that of hæmatein, but much weaker.

Iron-Brazilin (HICKSON, *Quart. Journ. Micr. Sci.*, xliv, 1901, p. 470) is better. Sections are mordanted for one to three hours in 1 per cent. sol. of iron alum in alcohol of 70 per cent. (made by dissolving 1 grm. of the salt in 23 c.c. of water, warm, and adding 77 c.c. of 90 per cent. alcohol after cooling), rinsed with alcohol, and put for three to sixteen hours into 0·5 per cent. sol. of Brazilin in alcohol of 70 per cent.

418. Orchella (Orseille), see WEDL (*Arch. path. Anat.*, lxxiv, p. 143) and FOL (*Lehrb.*, p. 192), and early editions of this work.

419. Orcein (ISRAEL, *Virchow's Archiv*, cv, 1886, p. 169 ; and *Prakticum der path. Hist.*, 2 Aufl., Berlin, 1893, p. 72) is a dye obtained from the lichen, *Lecanora parella* (tinctoria), and is not to be confused with *orcin*, another derivative of the same lichen. It is said to unite in itself the staining properties of the basic and acid stains, and also the combination of two contrast colours. ISRAEL stains sections in a solution containing 2 grm. of orcein,

2 grm. of glacial acetic acid, and 100 c.c. of distilled water, washes in distilled water, and passes rapidly through absolute alcohol to thick cedar oil, in which the preparations remain definitely mounted. Nuclei blue, protoplasm red.

Its principal use is for Unna's elastin stain.

See also " Connective Tissues " in Part II, and LAURENT, *Zeit. wiss. Mik.*, xiii, 1896, p. 302 ; RUZICKA, *ibid.*, xiv, 1898, p. 455 ; and WOLFF, *ibid.*, xix, 1903, p. 488.

420. **Purpurin**, see RANVIER'S *Traité technique*, p. 280 ; DUVAL'S *Précis de Technique histologique*, p. 221 ; and GRENACHER'S formula in *Arch. mik. Anat.*, xvi, 1879, p. 470. A very weak stain.

421. **Indigo.** Indigo is employed in histology in the form of solutions of so-called indigo carmine, or sulphindigotate of soda or potash. The simple aqueous solution gives a diffuse stain, but is of use when employed in conjunction with carmine. See below.

Thiersch's Oxalic Acid Indigo-Carmine (see *Arch. mik. Anat.*, i, 1865, p. 150).

422. **Other Vegetal Dyes.** See *early editions*. Those recommended by CLAUDIUS (*Zeit. wiss. Mik.*, xvii, 1900, p. 52) are superfluous.

CARMINE COMBINATIONS

423. **Seiler's Carmine followed by Indigo-Carmine** (*Am. Quart. Mic. Journ.*, i, 1879, p. 220). Stain in borax-carmine, wash out with HCl alcohol, wash out the acid, and after-stain in an *extremely dilute* alcoholic solution of indigo-carmine (2 drops of saturated aqueous solution added to an ounce of alcohol and filtered).

Lee found this method gave good results with sections, but not if it was attempted to stain in bulk.

424. **Merkel's Carmine and Indigo-Carmine in One Stain** (MERKEL, *Unters. anat. Anst. Rostock*, 1874 ; *Month. Mic. Journ.*, 1877, pp. 242 and 317).

Also NORRIS and SHAKESPEARE, *Amer. Journ. Med. Sci.*, January, 1877 ; MERKEL, *Mon. Mic. Journ.*, 1877, p. 242 ; MARSH, *Section Cutting*, p. 85 ; BAYERL, *Arch. Mik. Anat.*, xxiii, 1885, pp. 36, 37 ; MACALLUM, *Trans. Canad. Instit.*, ii, 1892, p. 222 ; *Journ. Roy. Mic. Soc.*, v, 1892, p. 698.

425. MAYER'S **Carmalum (or Hæmalum) and Indigo-Carmine in One Stain.** MAYER (*Mitth. Zool. Stat. Neapel*, xii, 1896, p. 320) obtains very good results by taking a solution of 0·1 grm. of indigo-carmine in 50 c.c. of distilled water, or 5 per cent. alum solution, and combining it with from 4 to 20 volumes of carmalum or hæmalum.

426. **Carmine and Picro-Indigo-Carmine** (RAMÓN Y CATAL, *Rev. de Sienc. med.*, 1895 ; CALLEJA, *Rev. trim. Microgr.*, ii, 1897, p. 101 ; *Zeit. wiss. Mik.*, xv, 1899, p. 323). For use after a carmine stain, RAMÓN takes a solution of 0·25 grm. of indigo-carmine in 100 grm. saturated aqueous solution of picric acid. Stain (*sections*) for five to ten minutes, wash in weak acetic acid, then in water, then remove the excess of picric acid with absolute alcohol, clear and mount.

Ramón also (*Elementos de Histologia*, 1897; quoted from *La Cellule*, xix, 1901, p. 212) employs the picro-indigo mixture after **Magenta**; stain strongly in saturated solution of magenta, rinse in water until no more colour comes away, and pass into the indigo mixture. See also Borrel, *Ann. Inst. Pasteur*, 1901, p. 57, or Lee et Henneguy, *Traité*, p. 268.

427. Carmine and Anilin Blue (or Bleu Lumière, or Bleu de Lyon) (Duval, *Précis de Technique Microscopique*, 1878, p. 225). Stain with carmine; dehydrate, and stain for a few minutes (ten minutes for a section of nerve-centres) in a solution of 10 drops of saturated solution of anilin blue in alcohol to 10 grm. of absolute alcohol. Clear with turpentine, without further treatment with alcohol, and mount in balsam.

Other authors recommend, instead of anilin blue, bleu de Lyon, dissolved in 70 per cent. alcohol acidulated with acetic acid (Maurice and Schulgin), or bleu lumière.

The solutions of both these colours should be extremely dilute for sublimate material, but strong for chrome-osmium material. It is possible to use them for staining in bulk.

Baumgarten (*Arch. mik. Anat.*, xl, 1892, p. 512) stains sections (of material previously stained in borax-carmine) for twelve hours in a 0·2 per cent. solution of bleu de Lyon in absolute alcohol, and washes out for about half that time before mounting in balsam. He recommends the process for cartilage and nerve-centres.

428. Carmine and Malachite Green. Maas (*Zeit. wiss. Zool.*, 1, 4, 1890, p. 527) recommends borax-carmine followed by weak alcoholic solution of malachite green, with a final washing out with stronger alcohol.

429. Carmine and Picro-nigrosin (Pianese). See *Journ. Roy. Mic. Soc.*, 1892, p. 292.

430. Carmine and Picric Acid. See § 266.

HÆMATEIN OR HÆMATOXYLIN COMBINATIONS

431. Hæmatoxylin and Picric Acid. See § 325.

432. Hæmatoxylin and Eosin. This popular combination gives results that are æsthetically beautiful, but (for most objects) is not so useful as many others, the eosin lacking in electivity. Objects may be stained with hæmatoxylin (either in the mass or as sections) and the sections stained for a few minutes in eosin. Lee thinks it is better to take the eosin weak, though it has been recommended (Stöhr, see *Zeit. wiss. Mik.*, i, 1884, p. 583) to take it saturated. Either aqueous or alcoholic solutions of eosin may be used.

Hickson (*Quart. Journ. Mic. Sci.*, 1893, p. 129) stains sections for one hour in a strong solution of eosin in 90 per cent. alcohol,

washes with alcohol, and stains for twenty minutes in a weak solution of hæmatoxylin.

This method is most particularly recommendable for embryological sections, as vitellus takes the eosin stain energetically, and so stands out boldly from the other germinal layers in which the blue of the hæmatoxylin dominates.

See also LIST (*Zeit. wiss. Mik.*, ii, 1885, p. 148); BUSCH (*Verh. Berl. Phys. Ges.*, 1887); GIERKE (*Zeit. wiss. Mik.*, i, 1884, p. 505).

Sections should be very well washed before being passed from eosin into hæmatoxylin or the reverse, as eosin very easily precipitates hæmatoxylin.

For the complicated and superfluous *mixtures* of RENAUT and of EVERARD, DEMOOR and MASSART, see FOL's *Lehrbuch*, p. 196, *Ann. Inst. Pasteur*, vii, 1893, p. 166, or *early editions*.

433. Hæmatoxylin and Congo. See § 332.

434. Hæmatoxylin and Safranin. RABL (*Morph. Jahrb.*, x, 1884, p. 215) stained *very lightly* with *very dilute* DELAFIELD's hæmatoxylin for twenty-four hours, then for some hours in (PFITZNER'S) safranin, and washed out with pure alcohol. The plasma stain is here given by the hæmatoxylin.

Similarly REGAUD, *Verh. Anat. Ges.*, xiv, 1900, p. 112.

FOÁ (*Festschr. Virchow*, 1891, p. 481) stains in a *mixture* of 25 c.c. of Böhmer's hæmatoxylin, 20 of 1 per cent. solution of safranin, and 100 of water for one to three minutes.

435. Hæmatoxylin and Säurefuchsin. Stain first with iron hæmatoxylin or hæmalum, then stain (sections) in 0·5 per cent. aqueous solution of Säurefuchsin, dehydrate and mount.

436. Hæmatoxylin and Säurefuchsin and Orange. Proceed as above, using for the second stain the following mixture: Säurefuchsin, 1 grm.; orange, 6 grm.; rectified spirit, 60 c.c.; water, 240 c.c. (from SQUIRE's *Methods and Formulæ*, p. 42). Using orange G (not mentioned by SQUIRE), we have had very good results.

The method of CAVAZZANI (*Riforma Med.*, Napoli, 1893, p. 604; *Zeit. wiss. Mik.*, xi, 3, 1894, p. 344) is far too complicated.

437. Hæmatoxylin and Picro-Säurefuchsin (VAN GIESON, *New York Med. Journ.*, 1889, p. 57; quoted from MOELLER, *Zeit. wiss. Mik.*, xv, 2, 1898, p. 172, which see for further details). Proceed as above, using for the second stain the picro-Säurefuchsin mixture, § 326. The second stain must not be too prolonged.

WEIGERT (*Zeit. wiss. Mik.*, xxi, 1904, p. 1) stains first in his iron-hæmatoxylin mixture (§ 284), rinses in water, and stains for a short time in his picro-Säurefuchsin (§ 326), rinses, dehydrates with 90 per cent. alcohol, and clears with carbolic acid-xylol mixture (1 : 3).

CHAPTER XXI

EXAMINATION AND PRESERVATION MEDIA

438. Introductory. We comprehend under this heading all the media in which an object may be examined to advantage.

All preservative media may be used for mounting, though the only media that will afford an *absolutely sure* preservation of soft tissues are the resinous ones.

439. Refractive Indices of Examination Media. An examination medium should be of such a *refractive index* as to afford a due degree of *visibility* of *colourless* (*unstained*) elements. The visibility of these is inversely as their transparency when penetrated by the medium. It is directly proportional to the *difference* between the *refractive indices* of the object and of the medium in which it is mounted. The greatest *transparency* is obtained when the refraction of the medium is the same as that of the tissue elements. Media having a lower index than that of the tissues give diminished transparency, but greater *visibility.* Media having a higher index than that of the tissues give great transparency, but *diminished visibility* of (unstained) details. Now the index of refraction of most tissue elements, after fixation and dehydration, is occasionally higher than that of Canada balsam : so that media of the greatest clearing power (*i.e.* giving the greatest transparency) must be looked for amongst reagents having an index superior to that of balsam, whilst for enhanced visibility of detail we must employ less refractive media, such as castor oil, glycerin, or water.

The following short list, extracted from BEHRENS' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten*, Braunschweig, 1892, p. 42, and other sources, may be useful as a guide to the *optical effects* of various media. The figures give the *approximate indices of refraction.* They should be accepted with some caution, on account of the variability of samples. The figures given for balsam refer evidently to the resin in the solid state and not to the solutions used for mounting, which are certainly much lower, according to the lower index of the solvent.

Air	1·000	Cedar-wood oil, not thickened	1·510
Methyl alcohol	1·323	Methyl benzoate	1·517
Distilled water	1·336	Crown glass	1·518
Sea water	1·343	Cedar-wood oil, thickened	1·520
Solution of white of egg	1·350	Gum damar	1·520
Absolute alcohol	1·367	Xylol balsam	1·524
Acetate of potash, saturated aqueous sol.	1·370	Oil of lemons	1·527
Glycerin with an equal quantity of water	1·397	Oil of cloves	1·533
Chloride of calcium, 90 per cent. in water	1·411	Canada balsam (solid)	1·535
Glycerin, Price's	1·460	Creosote	1·538
Oil of bergamot	1·464	Colophonium	1·545
Liquid paraffin	1·471	Carbolic acid	1·549
Olive oil	1·473	Oil of aniseed	1·557
Oil of turpentine	1·473	Oil of cinnamon (or cassia)	1·567
Glycerin, "concentrated"	1·473	Anilin oil	1·580
Gilson's Baume au Camsal	1·478	Sulphide of carbon	1·630
Gilson's Euparal	1·483	Tolu balsam	1·640
Terpinol	1·484	Monobromide of naphthalin	1·660
Castor oil	1·490	Solution of sulphur in sulphide of carbon	1·750
Xylol	1·497	Hyrax	1·82248

It will be seen that cedar oil has nearly the index of crown glass (this is true of the oil in the thick state to which it is brought by exposure to the air—not of the new, thin oil, which is less highly refractive); it therefore clears to about the same extent as Canada balsam. Clove oil has a much higher index, and therefore clears more than balsam; cinnamon oil higher still. Turpentine and bergamot oil have much lower indices, and therefore clear less. Note methyl benzoate as a thin substitute for cedar-wood oil for cover slip fresh mounts.

WATERY MEDIA *

440. Isotonic and "Indifferent" Liquids. The old distinction of "Indifferent" liquids, and those which have some action on tissues, appears to be misleading more than helpful; for *no* medium is without action on tissues except the plasma with which they are surrounded during the life of the organism; and this plasma itself is only "indifferent" whilst *in situ;* as soon as a portion of tissue is dissected out and transferred to a slide in a portion of plasma the conditions become artificial.

Water may be employed for the examination of structures that have been *well fixed;* but this is by no means applicable to the examination of fresh tissues. It is very far from being an "indifferent" liquid; many tissue elements are greatly changed by it (nerve-end structures, for instance), and some are totally

* These are again coming into use in cytology, especially for mounting fat stains, etc.

destroyed by its action if prolonged (for instance, red-blood corpuscles).

In order to render it inoffensive to fresh tissues it must have dissolved in it substances of similar diffusibility to those of the liquids of the tissue, so as to prevent the occurrence of osmosis, to which process the destructive action of pure water is mainly due. Now cell contents are a mixture of colloids and crystalloids ; consequently, in order to reduce osmotic processes to a minimum, it is necessary that the examination medium contain a due proportion of both crystalloids and colloids. By adding, for instance, white of egg to salt solution this end may be in some measure attained ; and, as a matter of fact, the liquids recommended as "indifferent" are generally found to contain both crystalloids and colloids. Liquids thus composed, in which tissue-elements are in osmotic equilibrium—that is, neither swell nor shrink—are said to be *isotonic* to the tissues ; whilst those in which they shrink are called *hypertonic*, and those in which they swell *hypotonic*. Solutions of common salt, in different concentrations, form the base of the most commonly employed isotonic liquids. For marine Invertebrates, sea-water is generally isotonic.

441. Salt Solution. See Addendum, p. 731, § 1430, for various useful media.

442. Pictet's **Liquid** (*Mitth. Zool. Stat. Neapel*, x, 1891, p. 89). Five to ten per cent. solution of chloride of manganese. These proportions are for *marine* animals, and for terrestrial animals will generally be found much too high. For these from 1 to 3 per cent. will be nearer the mark. Lee found this liquid excellent.

443. Aqueous Humour, Simple White of Egg. Require no preparation beyond filtering. They may be iodised if desired (see next §), or mixed with salt solution.

444. Iodised Serum. Max Schultze (*Virchow's Archiv.*, xxx, 1864, p. 263). We take the following from Ranvier (*Traité*, p. 76.

The only serum that gives really good results is the amniotic liquid of mammals. Flakes of iodine are added to it, and the flask frequently agitated for some days. The flask should have a wide bottom, so that the serum may form only a shallow layer in it.

Another method is as follows : Serum is mixed with a large proportion of tincture of iodine ; the precipitate that forms is removed by filtration, and there remains a strong solution of iodine in serum. This should be kept in stock, and a little of it added every two or three days to the serum that is intended for use. In general for maceration purposes a serum of a pale brown colour should be employed.

445. Artificial Iodised Serum (Frey, *Das Mikroskop*, 6 Aufl., 1877, p. 75). Distilled water 270 grm., white of egg 30, sodium chloride 2·5. Mix, filter, and add tincture of iodine.

446. Migula's **Glycerised Blood-serum** (see the paper in *Zeit. f. wiss. Mik.*, vii, 2, 1890, p. 172).

447. Chloride of Calcium (Harting, *Das Mikroskop*, 2 Aufl., p. 297). The aqueous solution, either saturated or diluted with 4 to 8 parts of water, has a low refractive index and does not dry up.

448. Acetate of Potash (MAX SCHULTZE, *Arch. mik. Anat.*, vii, 1872, p. 180). A nearly saturated solution in water. The index of refraction is lower than that of glycerin.

449. Syrup. A good strength is equal parts of loaf sugar and water. Dissolve by boiling. To preserve it from mould, chloral hydrate may be dissolved in it (1 to 5 per cent.)—Lee has used as much as 7 per cent., and found no disadvantage—or carbolic acid (1 per cent.).

It may be used as a mounting medium, but there is always risk of the sugar crystallising out.

FABRE-DOMERGUE (*Bull. Soc. Philomath.*, ix, 1899, p. 115) dissolves 200 parts of sugar in 400 of water, and adds 1 part of formaldehyde, and camphor to saturation.

450. Chloral Hydrate. Five per cent. in water (LADOWSKY, *Arch. f. mik. Anat.*, 1876, p. 359).

Or, 2·5 per cent. in water (BRADY, *British Copepods*).

Or, 1 per cent. in water (MUNSON, *Journ. Roy. Mic. Soc.*, 1881, p. 847).

MERCURIAL LIQUIDS

(*Lee gave these as examination media only, not as permanent mounting media. Media containing sublimate always end by making tissues granular.*)

451. GILSON'S **Fluid** (CARNOY'S *Biologie Cellulaire*, p. 94).

Alcohol of 60 per cent.	60 c.c.
Water	30 ,,
Glycerin	30 ,,
Acetic acid (15 parts of the glacial to 85 of water)	2 ,,
Bichloride of mercury	0·15 grm.

452. GAGE'S **Albumen Fluid** (*Zeit. f. wiss. Mik.*, 1886, p. 223).

White of egg	15 c.c.
Water	200 ,,
Corrosive sublimate	0·5 grm.
Salt	4 ,,

Mix, agitate, filter, and preserve in a cool place. Recommended for the study of red blood-corpuscles and ciliated cells.

453. PASINI'S **Fluids** (*Journ. de Mik.*, iv, 1880 ; *Journ. Roy. Mic. Soc.*, [N.S.] ii, 1882, p. 702, and early editions of this work). Antiquated and superfluous. They consist essentially of corrosive sublimate of from ½ to ⅓ per cent. strength, with the addition of a little salt or acetic acid.

454. GOADBY'S **Fluids** (*Micro. Dict.*, art. "Preservation," or early editions of this work). Quite unsuited for histological purposes.

OTHER FLUIDS *

455. Chloride and Acetate of Copper (RIPART et PETIT'S fluid, see § 95).

* For Berlese's gum chloral, see § 1188. This is an important medium because it does not contain glycerin, which attacks certain metallic impregnations, and which is the "active" constituent of most of these aqueous gum media. Notice that in all cases the gum should first be dissolved in the water, the other constituents being added later to the filtered liquid.

456. Tannin (CARNOY, *Biol. Cellulaire*, p. 95). Water 100 grm., powdered tannin 0·40 grm., as an examination medium only.

457. WICKERSHEIMER'S **Fluid** (*Zool. Anz.*, 1879, p. 670). Worthless for histological purposes.

458. Medium of Farrants (BEALE, *How to Work*, etc., p. 58). Picked gum arabic 4 oz., water 4, glycerin 2. See also the *Micrographic Dictionary*, and A. F. STANLEY KENT, in *Journ. Roy. Mic. Soc.*, 1890, p. 820.

459. Gum and Glycerin Medium (LANGERHANS, *Zool. Anzeig.*, ii, 1879, p. 575).

Gummi arab.	5·0
Aquæ	5·0

to which after twelve hours are added—

Glycerini	5·0
Sol. aquosa acid. corbal. (5·100)	10·0

460. ALLEN'S **Gum and Glycerin.** Prof. F. J. ALLEN (*in litt.*). Solution of gum arabic of the consistency of glycerin, strained, and $\frac{1}{8}$ volume of glycerin and $\frac{1}{20}$ of formol gradually incorporated. *Sets hard.*

461. HOYER'S **Gum with Chloral Hydrate or Acetate of Potash** (*Biol. Centralb.*, ii, 1882, pp. 23, 24). A high 60 c.c. glass with a wide neck is filled two-thirds full with gum arabic (in pieces), and then *either* a solution of chloral (of several per cent.) containing 5—10 per cent. of glycerin is added *or* officinal solution of acetate of potash or ammonia. Filter after solution. The solution with chloral is for carmine or hæmatoxylin objects—that with acetate for anilin objects.

462. COLE'S **Gum and Syrup Medium.** See last ed.

463. APÁTHY'S **Gum and Syrup Medium** (see § 511). This medium sets very hard and may also be used for ringing glycerin mounts.

464. FABRE-DOMERGUE'S **Glucose Medium** (*La Nature*, No. 823, 9 Mars, 1889, supp.). Glucose syrup diluted to 25° of the ærometer (sp. gr. 1·1968) 1,000 parts, methyl alcohol 200, glycerin 100, camphor to saturation. The glucose is to be dissolved in warm water, and the other ingredients added. The mixture, which is always acid, must be neutralised by the addition of a little potash or soda. It is said to preserve without change almost all *animal pigments*, but the mounts do not keep indefinitely.

465. BRUN'S **Glucose Medium** (from FABRE-DOMERGUE'S *Premiers Principes du Microscope*, 1889, p. 123). Distilled water 140 parts, camphorated spirit 10, glucose 40, glycerin 10. Mix the water, glucose, and glycerin, then add the spirit, and filter. HENNEGUY informed Lee that this liquid preserved the colour of preparations stained with anilin dyes, *methyl green included.*

466. Levulose is recommended by BEHRENS, KOSSEL u. SCHIEFFERDECKER (*Das Mikroskop, etc.*, 1889). It is uncrystallisable, and preserves well carmine and coal-tar stains (hæmatoxylin stains fade somewhat in it). The index of refraction is somewhat higher than that of glycerin. Objects may be brought into it out of water.

467. AMANN'S **Lactophenol** (from LANGERON, *C. R. Soc. Biol.*, lviii, 1905, p. 750). Carbolic acid, 20 ; lactic acid, 20 ; glycerin, 40 ; water, 20. For Nematodes, Acarids, etc. Add gradually drop by drop to the water containing the organisms. Not for mounting. Mount in glycerin jelly. (See also, *Amann, Zeit. f. wiss. Mik.*, 1896.)

GLYCERIN MEDIA *

468. **Glycerin.** Glycerin diluted with water is frequently employed as an examination and mounting medium. Dilution with water is sometimes advisable on account of the increased visibility that it gives to many structures. But for efficacious preservation undiluted glycerin, the strongest that can be procured, should be used (see BEALE, *How to Work*, etc.).

For closing glycerin mounts, the edges of the cover should first (after having been cleansed as far as possible from superfluous glycerin) be painted with a layer of *glycerin jelly;* as soon as this is set a coat of any of the usual cements may be applied. See next chapter.

Glycerin dissolves carbonate of lime, and is therefore to be rejected in the preparation of calcareous structures that it is wished to preserve.

469. **Extra-refractive Glycerin.** The already high index of refraction of glycerin (Price's glycerin, $n = 1{\cdot}46$) may be raised by dissolving suitable substances in it. Thus the refractive index of a solution of chloride of cadmium ($CdCl_2$) in glycerin may be 1·504 ; that of a saturated solution of sulphocarbonate of zinc in glycerin may be 1·501 ; that of a saturated solution of SCHERING's chloral hydrate (in crusts) in glycerin is 1·510 ; that of iodate of zinc in glycerin may be brought up to 1·56. For further details see *previous editions*, or *Journ. Roy. Mic. Soc.*, ii, 1879, p. 346 ; iii, 1880, p. 1051 ; (N.S.), i, 1881, pp. 943 and 366.

470. **Glycerin and Alcohol Mixtures.** These afford one of the best means of bringing delicate objects gradually from weak into strong glycerin. The object is mounted in a drop of the liquid, and left for a few hours or days, the mount not being closed. By the evaporation of the alcohol the liquid gradually increases in density, and after some time the mount may be closed, or the object brought into pure glycerin or glycerin jelly.

1. CALBERLA'S LIQUID (*Zeit. wiss. Zool.*, xxx, 1878, p. 442). Glycerin 1 part, alcohol 2, water 3.

2. We strongly recommend the following for very delicate objects : Glycerin 1 part, alcohol 1, water 2.

3. HANTSCH'S LIQUID. Glycerin 1 part, alcohol 3, water 2.

4. JÄGER'S LIQUID (VOGT and YUNG'S *Traité d'Anat. Comp. Prat.*, p. 16). Glycerin 1 part, alcohol 1, sea water 10.

* Refer to p. 600, § 1188, for directions for bringing objects into glycerin.

GLYCERIN JELLIES

471. **Glycerin Jellies** have a higher index than pure glycerin, and set hard enough to make luting unnecessary, though it is well to varnish the mount. To use them, you melt a small portion on a slide, introduce the object (previously soaked in water or glycerin), and cover.

Glycerin jelly mounts will last perfectly if properly sealed; some, now fifty-four years old, and in splendid condition, are in the possession of Mr. G. T. Harris of the Queckett Microscopical Club.

For preparing objects to mount in glycerin or jelly refer to § 1188, p. 600.

472. LAWRENCE'S **Glycerin Jelly** (DAVIES, *Preparation and Mounting of Microscopic Objects*, p. 84). Soak some gelatin for two or three hours in cold water, pour off the superfluous water, and heat until melted. To each fluid ounce of the gelatin, *whilst it is fluid but cool*, he adds a fluid drachm of the white of an egg. Boil until the albumen coagulates and the gelatin is quite clear, and to each ounce of the solution add 6 drachms of a mixture composed of 1 part of glycerin to 2 parts of camphor water.

473. BRANDT'S **Glycerin Jelly** (*Zeit. wiss. Mik.*, ii, 1880, p. 69). Melted gelatin 1 part, glycerin 1½ parts. The gelatin to be soaked in water and melted as above. After incorporating the glycerin, filter through spun glass pressed into the lower part of a funnel. He describes a simple arrangement for keeping the funnel warm during the filtering (see *early editions*). Some drops of carbolic acid should be added.

474. ZWEMER'S "**Glychrogel**" **Mounting Solution** is primarily intended for mounting frozen sections. It is made up as follows:—

Glycerin	20	c.c.
Gelatin, granulated	3	grm.
Chrome alum	0·2	,,
Distilled water	80	c.c.

Dissolve separately the chrome alum in 30 c.c. of water and the gelatin in 50 c.c. of water, using heat in both cases. Combine the glycerin with the gelatin solution while the latter is still warm and then, while stirring, add the warm chrome alum solution. After thoroughly mixing, filter and add a bit of camphor as a preservative. Keep the bottle well stoppered to prevent evaporation. To use, warm the solution in an oven to about 30° to 37° C., when it will flow freely.

475. KAISER'S **Glycerin Jelly** has been given § 180.

476. SQUIRE'S **Glycerin Jelly** (SQUIRE'S *Methods and Formulæ*, etc., p. 84). Soak 100 grm. of French gelatin in chloroform water, drain when soft, and dissolve with heat in 750 c.c. of glycerin. Add 400 c.c. of chloroform water with which has

been incorporated about 50 c.c. of fresh egg-albumen; mix thoroughly, and heat to boiling-point for about five minutes. Make up the total weight to 1550 c.c. with chloroform water. Filter in a warm chamber.

477. Heidenhain (*Zeit. wiss. Mik.*, xx, 1905, p. 328) takes of gelatin 9 parts, glycerin 7, and water 42, and to the filtrate adds drop by drop 14 parts of absolute alcohol.

478. Fischer (*Zeit. wiss. Mik.*, xxix, 1912, p. 65) takes 5 grm. of borax dissolved in 240 c.c. of water and adds 25 c.c. of glycerin. To this he adds 40 grm. of gelatin, dissolves with heat, and continues to heat gently until the solution has somewhat thickened. This remains fluid at ordinary temperatures.

479. Gilson's **Chloral Hydrate Jelly** (communicated by Gilson). One vol. of gelatin, melted *secundum artem*, and 1 vol. of Price's glycerin. Mix and add crystals of chloral hydrate until the volume has increased by one half; warm till dissolved. This gives a very *highly refractive* medium.

Geoffroy, *Journ. de Botan.*, 1893, p. 55 (see *Zeit. wiss. Mik.*, ix, 1893, p. 476), dissolves, by the aid of as little heat as possible, 3 to 4 grm. of gelatin in 100 c.c. of 10 per cent. aqueous solution of chloral hydrate.

HIGH REFRACTIVE LIQUIDS

480. Stephenson's **Biniodide of Mercury and Iodide of Potassium** (*Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, p. 167). A solution prepared by adding the two salts to water until each is in excess; the liquid will then be found to have a refractive index of 1·68. (If [Amann, *Zeit. wiss. Mik.*, xiii, 1896, p. 21] glycerin be taken instead of water, it rises to 1·78 or 1·80. Behrens [*Tabellen*, 1898, p. 71] takes biniodide 65 parts, iodide 50, and water 25. $n = 1{\cdot}71$). Any lower index can be obtained by suitable dilution with water. This fluid is very dense, its specific gravity being 3·02. It is highly antiseptic.

For marine animals a weak solution is probably well adapted, as about a 1 per cent. solution (5 minims to the ounce) will give the specific gravity of sea-water.

Covers should be sealed with white wax, and the mounts finished with two or three coatings of gold size and one of shellac.

Lee has experimented both with strong and with weak solutions. They are not adapted for the purposes of a *permanent* mounting medium, for the preparations are ruined by a precipitate which forms in the fluid. But as a temporary examination medium Lee occasionally found this solution valuable. Its optical properties are wonderful; it allows of the examination of watery tissues, *without any dehydration*, in a medium of refractive index surpassing that of any known resinous medium.

See further details in *early editions*.

481. **Monobromide of Naphthalin.** See *Journ. Roy. Mic. Soc.*, 1880, p. 1043 (Abbe and Van Heurck), and *Zool. Anz.*, 1882, p. 555 (Max Flesch).

RESINOUS MEDIA

482. **Resins and Balsams.** Resins and balsams consist of a vitreous or amorphous substance held in solution by an essential

oil. By distillation or drying in the air they lose the essential oil and pass into the solid state. It is these solidified resins that should be employed for microscopical purposes; for the raw resins always contain a certain proportion of water, which makes it difficult to obtain a clear solution with the usual menstrua, and is injurious to the optical properties of the medium and to the preservation of stains. All solutions should therefore be made by heating gently the balsam or resin in a stove until it becomes brittle when cold, and then dissolving in an appropriate menstruum.

Solutions made with volatile menstrua, such as xylol and chloroform, set rapidly, but become rapidly brittle. Solutions made with non-volatile media, such as turpentine, set much less rapidly, and pass much less rapidly into the brittle state.

Turpentine media preserve the *index of visability* of the preparations much longer than do media made with more volatile menstrua. Preparations made with these often become so transparent in course of time that much fine detail is often lost. (Such mounts may, however, be rejuvenated without removing the cover by putting them for a day or two into a tube of benzol: the benzol penetrates the balsam, and brings it down to a lower refractive index.)

For a permanent mounting medium of somewhat low index Lee unhesitatingly recommended *Euparal.* For cases in which a still lower index is desired, Gilson's *camsal balsam. Turpentine colophonium* is a safe and excellent medium, but is injurious to alum-hæmatein stains. For these, and in general where a strongly clearing medium is desired, *xylol balsam* is about the most recommendable, though it is not *perfectly* safe, the mounts sometimes developing granules. Seiler's *alcohol balsam* is a fine medium, and perfectly stable. *Oil of cedar* is sometimes useful, it keeps perfectly, and with time it thickens sufficiently to hold the cover in place; or if desired, preparations may be luted with Bell's cement.

483. Canada Balsam. Prepare with the solid balsam as described last §. The usual menstrua are xylol, benzol, chloroform, and turpentine. Turpentine has the advantages pointed out last §, but the defect that it does not always give a homogeneous solution with Canada balsam, as it does with colophonium. For most purposes *the xylol solution is the best.* If time be an object, a benzol solution should be preferred, as it sets much quicker than the xylol solution. The chloroform (and clove oil) solutions become very brown with age, and are *injurious to stains made with tar dyes.* Benzol is good when chemically pure and *free from water.*

Sahli (*Zeit. wiss. Mik.*, ii, 1885, p. 5) dissolves in cedar oil.

Apáthy (*Fauna Flora Golf. Neapel*, xxii, 1909, p. 18) takes balsam 2 parts, cedar oil (immersion) 1, and chloroform 1.

Samples of balsam that are *acid* * are frequently met with, and are injurious to some stains.

There seems to be a divergence of opinion among workers as to whether xylol or benzol is the better solvent for routine balsam. Dr. G. L. Alexander, for instance, writes to us that he considers xylol illogical because it has two methyl side groups to undergo oxidation. Mr. Muir, of the Edinburgh Pathology Laboratory, *always uses benzol, and has never had any serious trouble with fading.* We think that workers who have experienced fading with their xylol balsam should try benzol, which must, however, be pure and free from water.

To prevent the lid of the balsam bottle from sticking, C. M. Hector (Watson's *Microscope Record*, No. 21, 1930), uses a balsam bottle with a loose-fitting cap, places a ring of plasticine around the shoulder, and pushes the cap on to it. Corks are, of course, quite useless.

484. Seiler's **Alcohol Balsam** (*Proc. Amer. Soc. Mic.*, 1881, pp. 60–2; *Journ. Roy. Mic. Soc.* [N.S.], ii, 1882, pp. 126–7). Dissolve solid balsam in warm absolute alcohol, and filter through absorbent cotton. Objects may be mounted in it *direct from absolute alcohol.* We find it for most purposes admirable. It is *one of the most stable solutions known to us.* Care should be taken not to breathe on it, as this may cause cloudiness.

485. Damar (Gum Damar, or Dammar, or d'Ammar). The menstrua are the same as for balsam. We find xylol the best. For directions for preparing solutions, by various authors, see *early editions.* After ample experience we are convinced that *not one of these solutions can be depended on for permanent preservation.* Sooner or later, sometimes after a few weeks or days, or it may be only after months or years, granules make their appearance in the mounts. (See next §.)

486. Colophonium. A solution of pale colophonium in *oil of turpentine* keeps well and gives very good definitions. The solution should not be too thick, as it thickens with age.

This medium dries very slowly (so that ample time is afforded for arranging objects in it). In the winter a slide will take about a month before it will be hard enough to be safe with *oil-immersion lenses;* whereas an alcohol-balsam mount will be dry enough in a couple of days. It injures alum-hæmatein stains; as it sometimes develops clouds of globules it is not to be depended upon.

Dr. S. G. Scott of Oxford used both damar and colophonium instead of balsam, and very few of his preparations left after his decease are good. A large number have become granular.

Rehm (*Zeit. wiss. Mik.*, ix, 1893, p. 387) dissolves 1 part colophonium in 10 of *benzene.* Solutions in chloroform or xylol are also used by some, see Nissl in *Encycl. mik. Techn.*, ii, p. 274.

* There are various " neutral balsams " on sale now. We buy these for valuable slides, otherwise we use benzol balsam.

487. Venice Turpentine (VOSSELER, *Zeit. wiss. Mik.*, vi, 1889, pp. 292 *et seq.*). Commercial Venice turpentine is mixed in a tall cylinder glass with an equal volume of 96 per cent. alcohol, allowed to stand in a warm place for three or four weeks, and decanted. Preparations may be mounted in this medium direct from absolute alcohol. Celloidin sections can be mounted direct from 96 per cent. Stains keep well, according to VOSSELER, but MAYER finds hæmalum stains fade in it.

SUCHANNEK (*ibid.*, vii, 1896, p. 463) prepares it with equal parts of Venice turpentine and *neutral* absolute alcohol.

488. Thickened Oil of Turpentine has been used as a mounting medium by some workers. To prepare it, pour some oil into a plate, cover it lightly so as to protect it from dust without excluding the air, and leave it until it has attained a syrupy consistency.

489. GILSON'S **Sandarac Media** (*La Cellule*, xxiii, 1906, p. 427 : the formulæ have not been published, on account of the extreme difficulty of preparation. There are three of these. They are all of them solutions of gum Sandarac in " Camsal " and other solvents (" Camsal " is a liquid formed by the mutual solution of the two solids salol and camphor).

(1) **Camsal balsam (baume au camsal), propylic alcohol formula** ; a mixture of sandarac, camsal, and propylic alcohol, $n = 0{\cdot}478$.

(2) **Camsal balsam, isobutylic alcohol formula,** $n = 1{\cdot}485$.

(3) **Euparal,** a mixture of camsal, sandarac, eucalyptol, and paraldehyde, $n = 1{\cdot}483$. There are two sorts of this, the colourless and the green (" euparal vert "), the latter containing a salt of copper, which *intensifies hæmatoxylin stains.*

Objects may be prepared for mounting in camsal balsam by a bath of propylic or isobutylic alcohol and for euparal by a bath of the special solvent, " essence d'euparal." But this is *not necessary.* Objects may *always* be mounted *direct* from absolute alcohol, and even *at a pinch from alcohol of* 70 *per cent.* We generally prefer alcohol of 95 per cent. (absolute is dangerously volatile for sections). In difficult cases you may pass through a mixture of the medium and the solvent. Dr. ALEXANDER sometimes used paraldehyde containing some terpineol.

These media *work very kindly*, and do not dry too rapidly. Dr. G. A. ALEXANDER, like one of us, has experienced bad fading of hæmatoxylin preparations mounted in euparal. GATENBY noted years ago that euparal turned Ehrlich's hæmatoxylin slides a nasty colour, followed soon by fading. We cannot explain this phenomenon, but, as Dr. ALEXANDER points out to us (*in literis*), it does not occur with eosin-methylen blue preparations, and most of the coal-tar dyes seem to last well in euparal.

Dr. REES WRIGHT (*Ann. Trop. Med. & Parasit.*, vol. xxii, 1927) also states that he has found euparal invaluable for mounting blood films of the Romanowsky type, which have not faded for over six years. This author also uses euparal as a mountant for nematodes and insect genitalia, the objects being transferred from 70 per cent. alcohol to phenol, and when cleared to euparal.

The primary intention of these media is to spare delicate objects the usual treatment with absolute alcohol and essential oils. But they have another useful property—their *low index of refraction.* We find that that of euparal is just right for most delicate cytological researches, giving just the desired increase of visibility to unstained elements. Thus we frequently find that unstained spindles which are totally invisible in balsam become strongly visible in the most minute details in euparal. The camsal balsam, $n = 1{\cdot}478$, Lee has sometimes found valuable, but its index is a little too low for most things, and he generally used euparal. We consider that all the media which have been recommended on the score of a slightly lower index than balsam, such as damar, colophonium, Venice turpentine, castor-oil, are now superseded by these media.

490. Denham's **Sandarac Camphloral** (*Journ. Roy. Mic. Soc.*, 1923). Two parts by weight of crystalline chloral hydrate are ground in a glass mortar with one part of " flowers of camphor." If any difficulty is found in getting the last few crystals to dissolve the mixture is left in a warm place for a few hours. The liquid is then filtered. The sandarac solution is prepared separately. Selected crystals of the gum are dissolved in excess of isobutyl alcohol at 60° C. to make a solution thin enough to filter easily. It is then shaken up with animal charcoal, and filtered several times through paper, after which it is evaporated to a thick syrup, using a condenser to receive the alcohol. One part of gum is mixed with two of camphloral, and incorporated by warming and stirring. The product should have a refractive index of 1·485. Objects may be mounted from 70 per cent. alcohol. Euparal is commercially made in much the same way, the gum being dissolved in eucalyptol.

491. Sandarac (Lavdowsky, from *Ref. Handbook Med. Sci.*, Supp., p. 438). Gum sandarac 30 grs., absolute alcohol 50 c.c. Not trustworthy, the mounts scale badly.

492. Photographic Negative Varnish (for mounting large sections without cover-glasses). See Weigert, *Zeit. wiss. Mik.*, iv, 1887, p. 209.

493. Castor Oil. See Grenacher, *Abhandl. naturf. Ges. Halle-a.-S.*, Bd. xvi ; *Zeit. wiss. Mik.*, 1885, p. 244. Lee did not have good results with it.

494. Terpinol. $n = 1{\cdot}484$. See § 147.

495. Parolein (a pure form of paraffinum liquidum) is recommended by Coles (*Lancet*, 1911, p. 878) as being quite neutral and preserving certain coal tar stains. Ring mounts with Apáthy's gum syrup, § 511. Its index is 1·471, which I find too low for most things.

496. Cedar Oil. See § 482, *sub fin.*

497. Gum Thus, dissolved in xylol, is recommended by Eisen, *Zeit. wiss. Mik.*, xiv, 1897, p. 201.

498. Styrax and Liquidambar. See *Journ. Roy. Mic. Soc.*, 1883, p. 741 ; *ibid.*, 1884, pp. 318, 475, 655 and 827 ; and the places there quoted. Also *Bull. Soc. Belge de Mic.*, 1884, p. 178 ; and Fol, *Lehrb.*, p. 141. These are very highly refractive media, therefore seldom useful in histology.

499. Hyrax (DALLAS HANNA, *Journ. Roy. Mic. Soc.*, vol. l, 1930). A derivative of naphthalene. Soluble in benzene, xylol, but not in alcohol. Claimed by the inventor not to decompose or crystallise after several years. Refractive index 1·82248. Used for mounting diatoms.

F. J. BRISLEE (WATSON'S *Microscope Record*, No. 25, 1932), uses hyrax for diatoms, and finds it more suitable than balsam or styrax.

499 *bis*. Dioxan balsam (GATENBY, *Biological Technique*, 1937). Dioxan balsam appears to be reliable. Mount from dioxan or 96 % alcohol.

CHAPTER XXII

CEMENTS AND VARNISHES

500. Introduction. Two, or at most three, of the media given below will certainly be found sufficient for all useful purposes. For many years Lee had used only one cement (BELL'S). He recommended this both as a cement and varnish; gold size may be found useful for turning cells; and MILLER'S caoutchouc cement may be kept for occasions on which the utmost solidity is required. Marine glue is only necessary for making glass cells.

For the operations of mounting in fluids, and of making cells and ringing, see CARPENTER'S *The Microscope*.

CARPENTER lays great stress on the principle that the cements or varnishes used for fluid mounts should always be such as contain *no mixture of solid particles,* for those that do always become porous after a certain lapse of time. All fluid mounts *should have the edges of the cover carefully dried and be ringed with glycerin jelly before applying a cement; by this means all danger of running in is done away with.* See §§ 503 and 504. But no method yet devised will make a glycerin mount absolutely permanent.

See also AUBERT, *The Microscope,* xi, 1891, 150, and *Journ. Roy. Mic. Soc.*, 1891, p. 692; BECK, *The Microscope,* xi, 1891, pp. 338, 368, and *Journ. Roy. Mic. Soc.*, 1892, p. 293; BEHRENS' *Tabellen zum Gebrauch bei mikroskopischen Arbeiten* (Bruhn, Braunschweig, 1892); ROUSSELET, *Journ. Quek. Mic. Club,* vii, 1898, p. 93; and as to the comparative *tenacity* of divers cements, BEHRENS, *Zeit. wiss. Mik.*, ii, 1885, p. 54, and AUBERT, *Amer. Mon. Mic. Journ.*, 1885, p. 227; *Journ. Roy. Mic. Soc.*, 1886, p. 173. AUBERT places MILLER'S caoutchouc cement at the head of the list, LOVETT'S cement coming half-way down, and zinc white cement at the bottom, with less than one-quarter the tenacity of the caoutchouc cement.

501. Peter Gray's Sealing Medium. For glycerin and other mounts: 4 parts anhydrous lanolin, 8 parts resin, 1 part dry Canada balsam. Melt together. This forms a solid mass on cooling. For circular cover-glasses a piece of metal tube of the necessary diameter is heated over a Bunsen, dipped into the molten mixture and applied to the cover-glass. For square covers, a broad bent needle is used. (WATSON'S *Microscope Record,* No. 33, 1934).

502. Paraffin. *Temporary* mounts may be closed with paraffin, or white wax, by applying it with a bent wire, and be made more or less permanent by varnishing.

503. Gelatin Cement (MARSH'S *Section-cutting,* 2nd ed., p. 104). Take half an ounce of NELSON'S opaque gelatin, soak well in

water, melt in the usual way, stir in 3 drops of creosote. It is used warm.

When the ring of gelatin has become quite set and dry, it may be painted over with a solution of bichromate of potash made by dissolving 10 gr. of the salt in an ounce of water. This should be done in daylight, in order to render the gelatin insoluble. The cover may then be finished with BELL's cement. This process is particularly adapted for glycerin mounts.

504. The Paper Cell Method. By means of two punches Lee cut out rings of paper of about a millimetre in breadth, and of about a millimetre smaller in diameter than the cover-glass. *Moisten* the paper ring with mounting fluid, and centre it on the slide. Fill the cell thus formed with mounting fluid; arrange the object in it; put the cover on; fill the annular space between the paper and the margin of the cover with glycerin jelly (a turn-table may be useful for this); and as soon as the gelatin has set turn a ring of gold-size on it, and when that is quite dry, varnish with BELL's cement.

For greater safety, the gelatin may be treated with bichromate, according to MARSH's plan, last §.

505. ROUSSELET's **Method for Aqueous Mounts** (*op. cit.*, § 500). Close the mount with a ring of a mixture of 2 parts of a solution of damar in benzol and 1 part gold-size. When dry, put on three or four thin coats of pure gold-size at intervals of twenty-four hours, and finish with a ring of WARD's brown cement.

506. MILLER's **Caoutchouc Cement.** Composition unknown. May be obtained from the opticians. A very tenacious and quickly drying cement. It may be diluted by a mixture of equal parts of chloroform and strong alcohol (see ROUSSELET, *Journ. Quek. Club*, v, ii, 1895, p. 8).

507. Asphalt Varnish (*Bitume de Judée*). Unquestionably one of the best of these media, either as a cement or a varnish, *provided it be procured of good quality*. It can be obtained from the opticians.

508. Brunswick Black. See *early editions*, or BEALE, *How to Work*, etc., p. 49.

509. Gold Size. Best obtained from the opticians. It is soluble in oil of turpentine. A good cement, *when of good quality*, and very useful for turning cells.

510. Turpentine, Venice Turpentine (CSOKOR, *Arch. mik. Anat.*, xxi, 1882, p. 353; PARKER, *Amer. Mon. Mik. Journ.*, ii, 1881, pp. 229—30). Venice turpentine, or common resinous turpentine, evaporated by heat until brittle on cooling. It is used for closing glycerin mounts in the following manner: Square covers are used, and superfluous glycerin is cleaned away from the edges in the usual way. The cement is then put on with a piece of wire bent at right angles; the short arm of the wire should be just the length of the side of the cover-glass. The wire is heated in a spirit lamp,

plunged into the cement, some of which adheres to it, and then brought down flat upon the slide at the margin of the cover. The turpentine distributes itself evenly along the side of the cover, and hardens immediately, so that the slide may be cleaned as soon as the four sides are finished. It is claimed for this cement that it is perfectly secure and never runs in. It sets hard in a few seconds.

511. APÁTHY's **Cement for Glycerin Mounts** (*Zeit. wiss. Mik.*, vi, 1889, p. 171). Equal parts of hard (60° C. melting-point) paraffin and Canada balsam. Heat together in a porcelain capsule until the mass takes on a golden tint and no longer emits vapours of turpentine. Used by warming and applying with a glass rod or brass spatula. One application is enough. Does not run in, and never cracks.

512. **Canada Balsam, or Damar.** Cells are sometimes made with these. They are elegant, but in my experience are not reliable for permanent mounts.

513. **Tolu Balsam Cement** (CARNOY's *Biol. Cell.*, p. 129). Tolu balsam, 2 parts, Canada balsam 1, saturated solution of shellac in chloroform, 2 parts. Add enough chloroform to bring the mixture to a syrupy consistence. Carnoy finds this cement superior to all others.

514. For the cements of WARD, BELL and CLARKE and for KRÖNIG's Colophonium and Wax, Marine Glue, Amber and Copal, and Sealing Wax Varnish, see *last edition.*

PART II

SPECIAL METHODS AND EXAMPLES

CHAPTER XXIII

INJECTION—GELATIN MASSES (WARM)

515. Introduction. Injection masses are composed of a coloured substance called the *colouring mass*, and of a substance with which that is combined called the *vehicle.*

For instructions as to the operation of injecting, and the necessary apparatus, see *The Micrographic Dictionary*, RUTHERFORD'S and SCHÄFER'S *Practical Histology*, the treatises of ROBIN and RANVIER, BEALE'S *How to Work with the Microscope*, the *Lehrbuch der vergleichenden Mikroscopischen Anatomie* of FOL, and (for apparatus especially) the article in the *Enzyk. d. mik. Technik.* For injections for the study of the angiology of Vertebrates the practice of Robin and Ranvier may safely by followed. For injections of Invertebrates (and indeed, for vertebrates if it is desired to demonstrate the minute structure of environing tissues at the same time as the distribution of vessels) masses not containing gelatin are generally preferable to gelatin masses ; and we would recommend as particularly convenient the Prussian blue glycerin masses of BEALE. Glycerin masses have the great advantage that they are used *cold.*

All formulæ which only give opaque masses, or are only suitable for coarse injections for naked eye study, have been suppressed.

In § 809 is a section on injection of embryos.

516. Vaso-dilators. In order that an injection may run freely it is necessary that the vessels of the subject be in a relaxed state. To this end the older anatomists used to wait until *rigor mortis* had passed off before injecting. But it is evidently preferable in the interest of the proper preservation of the tissues to inject before *rigor mortis* has set in. Unfortunately, when this is done, it is found that most injection masses—glycerin masses especially —stimulate the contraction of the vessels, so that frequently it is very difficult to get in the injection. In these cases it may be advisable to use a vaso-dilator. The animal may be anæsthetised with a mixture of ether and *nitrite of amyl*, and finally killed with pure nitrite. Or, after killing before the injection mass is thrown in. In any case it is advisable to add a little nitrite to the mass just before using. The relaxing power is very great (see OVIATT and SARGENT, in *St. Louis Med. Journ.*, 1886, p. 207 ; and *Journ. Roy. Mic Soc.*, 1887, p. 341).

Bayliss (in personal communication) suggests for prevention of coagulation, to wash out in citrate of soda (4 per cent.) instead of 0·75 NaCl, or to add ½ per cent. oxalate of calcium to 0·75 per cent. NaCl. To relax arterial walls, add sodium nitrite 1 in 500 to the washing out fluid.

Or, *morphia* may be added to the injection mass, or 1 per cent. of *lactic acid.* Mozejko (*Zeit. wiss. Mik.*, xvi, 1909, p. 545) prefers a saturated solution of neutral *Peptonum siccum*, which has the advantage of hindering coagulation. For warm-blooded animals the mass should be warmed to body-temperature; and in all cases masses that tend to dehydrate tissues should be avoided if possible.

ROBIN'S MASSES

517. Robin's **Gelatin Vehicle** (*Traité*, p. 30). One part of gelatin soaked and melted in 7, 8, 9, or even 10 parts of water, on a water-bath.

This vehicle, like all gelatin masses, is liable to be attacked by mould if kept long; camphor and carbolic acid do not suffice to preserve it. Chloral hydrate 2 per cent. is said to do so.

518. Robin's **Glycerin-Gelatin Vehicle** (*Traité*, p. 32). Dissolve in a water-bath 50 grm. of gelatin in 300 grm. of water, in which has been dissolved some arsenious acid; add of glycerin 150 grm., and of carbolic acid a few drops. Unlike the pure gelatin vehicles, this mass does keep indefinitely.

Frankl (*Zeit. f. wiss. Zool.*, lxiii, 1897, p. 28) prepares a similar vehicle, and adds to it a little solution of corrosive sublimate and a crystal of thymol.

519. Robin's **Carmine Colouring Mass** (*Traité*, p. 33). Rub up 3 grm. of carmine with a little water and enough ammonia to dissolve it. Add 50 grm. of glycerin and filter.

Take 50 c.c. of glycerin with 5 c.c. of acetic acid, and add it by degrees to the carmine-glycerin, until a slightly acid reaction is obtained (as tested by very sensitive blue test-paper, moistened and held over the mixture).

One part of this mixture is to be added to 3 or 4 parts of the vehicles given above.

520. Robin's Ferrocyanide of Copper Colouring Mass (*ibid.*, p. 34). Take—

(1) Ferrocyanide of potassium (concentrated solution)	20 c.c.
Glycerin	50 ,,
(2) Sulphate of copper (concentrated solution)	35 ,,
Glycerin	50 ,,

Mix (1) and (2) slowly, with agitation; at the moment of injecting combine with 3 volumes of vehicle.

521. Robin's **Prussian Blue Colouring Mass** (*ibid.*, p. 35, and 2nd ed., p. 1013).

Take—

(A)	Ferrocyanide of potassium (sol. sat.) . .	90 c.c.
	Glycerin	50 ,,
(B)	Solution of ferric chloride at 30° Baumé .	3 ,,
	Glycerin	50 ,,

Mix slowly and combine the mixture with 3 parts of vehicle. It is well to add a few drops of HCl.

CARMINE-GELATIN MASSES

522. Ranvier's **Carmine-Gelatin Mass** (*Traité technique*, p. 116). Take 5 grm. Paris gelatin, soak until quite swollen and soft, wash, drain and melt it in the water it has absorbed over a water-bath. When melted add slowly, and with continual agitation, $2\frac{1}{2}$ grm. of carmine rubbed up with a little water, and just enough ammonia, added drop by drop, to dissolve the carmine into a *transparent* solution.

The mixture is now neutralised by adding cautiously, drop by drop, with continual agitation, a solution of 1 part of glacial acetic acid in 2 parts of water. (When the mass is near neutrality, dilute the acetic acid still further.) The instant of saturation is determined by the smell of the solution, which gradually changes from ammoniacal to sour. As soon as the sour smell is perceived the liquid must be examined under the microscope. If it contains a granular precipitate of carmine, too much acid has been added, and it must be thrown away.

The mass, having been perfectly neutralised, is strained through new flannel.

523. How to Neutralise a Carmine Mass (Ville, *Gaz. hebd. d. Sci. méd. de Montpellier*, Fév., 1882). Ville points out that when carmine is treated with ammonia a certain proportion of the ammonia combines with the carmine and the rest remains in excess. It is this *excess* that it is required to neutralise precisely, not the *whole* of the ammonia employed.

To neutralise the acidity of commercial gelatin, it should be washed for an hour or so in running water.

As to the neutralisation of the colouring mass Ville is of opinion that the sour smell cannot be safely relied on in practice, and prefers to employ dichroic litmus paper (litmus paper sensitised so as to be capable of being used equally for the demonstration of acids and bases). For directions for preparing this see *loc. cit.* or *previous editions.*

524. Hoyer's **Carmine-Gelatin Mass** (*Biol. Centralb.*, 1882, p. 21). Take a concentrated gelatin solution and add to it the

needful quantity of neutral carmine staining solution (*loc. cit.*, p. 17). Digest in a water-bath until the dark violet-red colour begins to pass into a bright red tint. Then add 5 to 10 per cent. by volume of glycerin, and at least 2 per cent. by weight of chloral, in a concentrated solution, and strain.

525. FOL'S **Carmine-Gelatin Mass** (*Lehrb.*, p. 13). This can be *kept in the dry state* for an indefinite length of time.

Gelatin in sheets is cut into strips which are macerated for two days in carmine solution (prepared by diluting one volume of strong ammonia with three of water and adding carmine to *saturation*, and filtering after a day or two). The strips are then rinsed and put for a few hours into water acidulated with acetic acid, then washed on a sieve for several hours in running water, dried on parchment paper, or on a net, and preserved for future use. To get the mass ready for use, the strips are soaked for an hour in water and melted on a water-bath in 10 to 20 parts of water.

For another process, which is said to give somewhat better results, but is more complicated, see *loc. cit.*, or *Zeit. wiss. Zool.*, xxxviii, p. 492, or *previous editions*.

526. KRAUSE'S **Carmine-Gelatin Mass** (*Zeit. wiss. Mik.*, xxvi, 1909, p. 1). 100 grm. gelatin soaked in water, put for two to three days into a solution of 15 grm. carmine in 2 litres of water with 100 grm. of borax, washed, treated for a short time with hydrochloric acid of 2 per cent., washed, melted and preserved with camphor.

527. Other Carmine-Gelatin Masses. THIERSCH'S, see *Arch. mik. Anat.*, 1865, p. 148. GERLACH'S, see RANVIER, *Traité*, p. 118, CARTER'S, see BEALE, p. 113. DAVIES, see his *Prep. and Mounting of Mic. Objects*, p. 138.

BLUE GELATIN MASSES

528. RANVIER'S **Prussian Blue Gelatin Mass** (*Traité*, p. 119). Make a concentrated solution of ferric sulphate in distilled water, and pour it *gradually* into a concentrated solution of yellow prussiate of potash. There is produced a precipitate of insoluble Prussian blue. Wash this on a felt strainer, underneath which is arranged a paper filter in a glass funnel, for some days, until the liquid begins to run off blue from the second filter. The Prussian blue has now become soluble. The strainer is turned inside out and agitated in distilled water; the Prussian blue will dissolve if the quantity of water be sufficient.

The solution may now be injected just as it is, or it may be kept in bottles till wanted, or evaporated in a stove, and the solid residuum put away in bottle.

For injections, if a simple aqueous solution be taken, it should be *saturated.* Such a mass never transudes through the walls of vessels. Or it may be combined with one-fourth of glycerin, or with one twenty-fifth of gelatin soaked for an hour in water and melted over a water-bath in the water it has absorbed. The

gelatin is to be poured gradually into the Prussian blue, on the water-bath, stirring continually until the curdy precipitate that forms at first has disappeared. Filter through new flannel and keep at 40° C. until injected.

529. Brücke's **Soluble Berlin Blue** (*Arch. mik. Anat.*, 1865, p. 87). Make a solution of ferrocyanide of potassium containing 217 grm. of the salt to 1 litre of water, and one of 1 part commercial chloride of iron in 10 parts water. Take equal volumes of each, and add to each of them twice its volume of a cold saturated solution of sulphate of soda. Pour the chloride solution into the ferrocyanide solution, stirring continually. Wash the precipitate on a filter until soluble, dry it, press between blotting-paper in a press, break the mass in pieces, and dry in the air.

The concentrated solution of the colouring matter is to be gelatinised with just so much gelatin that the mass forms a jelly when cold. For another method, see *previous editions*.

530. **Other Blue Gelatin Masses.** Hoyer's, *Arch. mik. Anat.*, 1876, p. 649; Guignet's, *Journ. de Microgr.*, 1889, p. 94; *Journ. Roy. Mic. Soc.*, 1889, p. 463; Thiersch's, *Arch. mik. Anat.*, i, 1865, p. 148; Fol's, *Zeit. wiss. Zool.*, xxxviii, 1883, p. 494; and *previous editions*.

OTHER COLOURS

531. Hoyer's **Silver Nitrate Yellow Gelatin Mass** (*Biol. Centralb.*, ii, 1882, pp. 19, 22). A concentrated solution of gelatin is mixed with an equal volume of a 4 per cent. solution of nitrate of silver and warmed. To this is added a very small quantity of an aqueous solution of pyrogallic acid, which reduces the silver in a few seconds; chloral and glycerin are added as directed § 524.

This mass is yellow in the capillaries and brown in the larger vessels.

532. **Other Colours.** Hoyer's **Green** (*Biol. Centralb.*, ii, 1882, p. 19). Made by mixing a blue mass and a yellow mass. Thiersch's **Green** (*Arch. mik. Anat.*, 1865, p. 149). Robin's Scheele's **Green** (Robin, *Traité*, p. 37). Harting's **White** (see Frey, *Le Microscope*, p. 190). Frey's **White** (*ibid.*). Teichmann's **White** (*ibid.*, p. 191). Fol's **Brown** (*Zeit. wiss. Zool.*, xxxviii, 1883, p. 494). Miller's **Purple** (see *Amer. Mon. Mic. Journ.*, 1888, p. 50; *Journ. Roy. Mic. Soc.*, 1888, p. 518). Fol's **Lead Chromate** (*Lehrb.*, p. 15). Robin's **Cadmium** (his *Traité*, p. 36). Thiersch's **Lead Chromate** (*Arch. mik. Anat.*, 1865, p. 149). Hoyer's **Lead Chromate** (*ibid.*, 1867, p. 136); or, for any of these, see *early editions*.

533. Ranvier's **Gelatin Mass for Impregnation** (*Traité*, p. 123). Concentrated solution of gelatin, 2, 3, or 4 parts; 1 per cent. nitrate of silver solution, 1 part.

Neuville (*Ann. Sci. Nat.*, xiii, 1901, p. 36) takes a solution of 10 grm. of soaked gelatin in 100 c.c. of 1 per cent. solution of nitrate of silver.

534. Friendethal's **Hardening Mass** (*Centralb. Phys.*, xii, 1899, p. 267). A 10 per cent. solution of gelatin, combined with a colouring mass, and with 1 volume of 4 per cent. formol, serves for injecting vessels and hardening the tissues at the same time.

CHAPTER XXIV

INJECTIONS—OTHER MASSES (COLD)

535. Fol's **Metagelatin Vehicle** (*Lehrb.*, p. 17). If a slight proportion of ammonia be added to a solution of gelatin, and the solution be heated for several hours, the solution passes into the state of metagelatin, that is, a state in which it no longer coagulates on cooling and can be injected without warming. Colouring masses may be added to this vehicle, which may also be thinned by the addition of weak alcohol. After injection the preparations are thrown into strong alcohol or chromic acid, which sets the mass.

According to the *Enzyk. mik. Technik.*, metagelatin is usually prepared by warming with concentrated acetic or oxalic acid. It may be neutralised afterwards with carbonate of lime.

Tantler's **Gold Gelatin Mass** (*Zeit. wiss. Mik.*, xviii, 1901, p. 22). Five grm. of gelatin are soaked in 100 c.c. of water, warmed and melted, and combined with Berlin blue. Then 5 to 6 grm. of iodide of potassium are slowly incorporated. The mass generally remains liquid enough for injection down to a temperature of 17° C., but if it should coagulate a little more iodide should be added. After injection you may fix with 5 per cent. formol. The specimens will bear decalcification with hydrochloric or sulphurous acid.

Pearl (*Journ. Appl. Micr.*, v, 1902, p. 1736) takes 8 to 10 per cent. of the iodide.

Mayer (*Grundzüge*, Lee and Mayer, 1910, p. 250) takes simply 10 grm. gelatin, 10 grm. *hydrate of chloral* and 100 c.c. water.

Mozejko (*Zeit. wiss. Mik.*, xxvii, 1910, p. 374) finds that 10 per cent. (or more) of sodium salicylate will retard the setting of gelatin for hours at normal temperatures.

Any of these masses may be made to set in the tissues by means of weak formol.

GLYCERIN MASSES

536. Beale's **Carmine Glycerin Mass** (*How to Work*, etc., p. 95). Five grains of carmine are dissolved in a little water with about 5 drops of ammonia, and added to half an ounce of glycerin. Then add half an ounce of glycerin with 8 or 10 drops of acetic or hydrochloric acid, gradually, with agitation. Test

with blue litmus paper, and if necessary add more acid till the reaction is decidedly acid. Then add half an ounce of glycerin, two drachms of alcohol, and six drachms of water. We have found this useful, but not so good as the two following.

537. Beale's **Prussian Blue** (*How to Work*, etc., p. 93).

Common glycerin	1 ounce.
Spirits of wine	1 ,,
Ferrocyanide of potassium . . .	12 grains.
Tincture of perchloride of iron . .	1 drachm.
Water	4 ounces.

Dissolve the ferrocyanide in one ounce of the water and glycerin, and add the tincture of iron to another ounce. These solutions should be mixed together *very gradually*, and well shaken in a bottle, *the iron being added to the solution of the ferrocyanide of potassium*. Next the spirit and the rest of water are to be added very gradually, the mixture being constantly shaken.

Injected specimens should be preserved in acidulated glycerin (*e.g.* with 1 per cent. acetic acid), otherwise the colour may fade.

538. Beale's **Acid Prussian Blue** (*ibid.*, p. 296).

Price's glycerin	2 fluid ounces.
Tinct. of sesquichloride of iron .	10 drops.
Ferrocyanide of potassium . .	3 grains.
Strong hydrochloric acid . .	3 drops.
Water	1 ounce.

Proceed as before, dissolving the ferrocyanide in one half of the glycerin, the iron in the other, and adding the latter drop by drop to the former. Finally add the water and HCl. Two drachms of alcohol may be added to the whole if desired.

We find this excellent.

539. Ranvier's **Prussian Blue Glycerin Mass** (*Traité*, p. 120). The Prussian blue fluid, § 537, mixed with one fourth of glycerin.

540. Thoma's **Indigo-Carmine** (*Arch. Anat. Phys., Anat. Abth.*, 1899, p. 270). Dissolve 0·15 grm. sulphindigotate of soda in 50 c.c. water, filter, add 40 c.c. glycerin and gradually, with agitation, 10 c.c. of a filtered 10 per cent. solution of sodium chloride in water. If desired, 3 c.c. of a 1 per cent. solution of morphia may be added to dilate arteries. A fine precipitate is formed, which is injected with the mass.

541. **Gamboge Glycerin** (Harting, *Das Mikroskop*, 1866, 2, Theil, p. 124). Gamboge rubbed up with water and added to glycerin; or a saturated alcoholic solution of gamboge added to a mixture of equal parts of glycerin and water. Any excess of alcohol may be got rid of by allowing the mass to stand for twenty-four hours.

542. **Other Colours.** Any of the colouring masses, §§ 528 to 532, or other suitable colouring masses, combined with glycerin, either dilute or pure.

PURELY AQUEOUS MASSES. (See also § 809)

543. Ranvier's **Prussian Blue Aqueous Mass** (*Traité*, p. 120). The soluble Prussian blue, injected without any vehicle. It does not extravasate.

544. Müller's **Berlin Blue** (*Arch. mik. Anat.*, 1865, p. 150). Precipitate a concentrated solution of Berlin blue by means of ½ to 1 volume of 90 per cent. alcohol. The precipitate is very finely divided; and the fluid may be injected at once.

545. Mayer's **Berlin Blue** (*Mitth. Zool. Stat. Neapel*, 1888, p. 307). A solution of 10 c.c. of tincture of perchloride of iron in 500 c.c. of water is added to a solution of 20 grm. of yellow prussiate of potash in 50 c.c. of water, allowed to stand for twelve hours, decanted, the deposit washed with distilled water on a filter until the washings come through dark blue (one to two days), and the blue dissolved in about a litre of water. It is well to add a little acetic acid and to put up the objects in an acid liquid.

546. Emery's **Aqueous Carmine** (*ibid.*, 1881, p. 21). To a 10 per cent. ammoniacal solution of carmine is added acetic acid, with continual stirring, until the colour of the solution changes to blood-red. The supernatant clear solution is injected cold without further preparation. The injected organs are thrown at once into strong alcohol to fix the carmine. For injection of fishes.

547. Taguchi's **Indian Ink** (*Arch. mik. Anat.*, 1888, p. 565). Chinese or (better) Japanese ink well rubbed up on a hone until a fluid is obtained that does not run when dropped on thin blotting-paper, nor form a grey ring round the drop. Inject until the preparation appears quite black, and throw it into some hardening liquid (not pure water).

Della Rosa (*Ver. Anat. Ges.*, 1900, p. 141) recommends the liquid Chinese ink sold in the shops.

PARTIALLY AQUEOUS MASSES

548. Joseph's **White-of-Egg** (*Ber. naturw. Sect. Schles. Ges.*, 1879, pp. 36—40; *Journ. Roy. Mic. Soc.*, ii, 1882, p. 274). "Filtered white-of-egg, diluted with 1 to 5 per cent. of carmine solution. . . . This mass remains liquid when cold, coagulates in dilute nitric acid, chromic or osmic acid, and remains transparent in the vessels." For invertebrates.

Grosser (*Zeit. wiss. Mik.*, xvii, 1900, p. 178) rubs up Indian ink with white-of-egg; Hoffmann (*Zeit. Morph. Anthrop.*, iii,

1901, p. 240) with blood-serum ; so also HAMBURGER, *Zeit. wiss. Mik.*, xxv, 1908, p. 1 (2 vols. of the ink—" Perltusche "—to 3 of serum).

549. BJELOUSSOW'S **Gum Arabic Mass** (*Arch. Anat. Phys.*, 1885, p. 379). Make a syrupy solution of gum arabic and a saturated solution of borax in water. Mix the solutions in such proportions as to have in the mixture 1 part of borax to 2 of gum arabic. Rub up the transparent, almost insoluble mass with distilled water, added little by little, then force it through a fine-grained cloth. Repeat these operations until there is obtained a mass that is free from clots. It should then coagulate in the presence of alcohol, undergoing at the same time a dilatation to twice its original volume. The vehicle thus prepared may be combined with any colouring mass except cadmium and cobalt.

After injection the preparation is thrown into alcohol, and the mass sets immediately, swelling up as above described and consequently showing vessels largely distended.

Cold-blooded animals may be injected whilst alive with this mass. It does not flow out of cut vessels. Injections keep well in alcohol. If it be desired to remove the mass from any part of a preparation, this is easily done with dilute acetic acid.

550. **Milk** has been recently recommended by FISCHER (*Centralb. allg. Path.*, xiii, 1902, p. 277 ; *Zeit. wiss. Mik.*, xx, 1903, p. 224). It runs well, does not extravasate, and can be used for auto-injection of the living subject.

After injection it should be coagulated by putting the organs for at least twenty-four hours into a mixture of 75 parts of formol, 15 of acetic acid, and 1000 of water (pure formol will not do). They are then sectioned, and the sections stained with Sudan III or Scharlach R, which stain the milk. They cannot be mounted in balsam.

CELLOIDIN AND OTHER MASSES

551. SCHIEFFERDECKER'S **Celloidin Masses** (*Arch. Anat. Phys.*, 1882 [*Anat. Abth.*], p. 201). (For Corrosion preparations.) See *previous editions* ; HOCHSTETTER'S **Modification of** SCHIEFFERDECKER'S **Mass** (*Anat. Anz.*, 1886, p. 51) ; BUDGE'S **Asphaltum Mass** (*Arch. mik. Anat.*, xiv, 1877, p. 70), or *early editions* ; HOYER'S **Shellac Mass** (*Arch. mik. Anat.*, 1876, p. 645). For this and that of BELLARMINOW (*Anat. Anz.*, 1888, p. 605), see *early editions* ; HOYER'S **Oil-colour Masses** (*Internat. Monatsschr. Anat.*, 1887, p. 341) ; SEVEREANU'S, *Verh. Anat. Ges.*, 21 *vers*, 1906, p. 275 ; PANSCH'S **Starch Mass** (*Arch. Anat. Entw.*, 1877, p. 480 ; 1880, pp. 232, 371 ; 1881, p. 76 ; 1882, p. 60 ; 1883, p. 265 ; and a modification of the same by GAGE, *Amer. Mon. Mic. Journ.*, 1888 p. 195) ; TEICHMANN'S **Linseed-Oil Masses** (*S. B. Math. Kl. Krakau Akad.*, vii, pp. 108, 158 ; *Journ. Roy. Mic. Soc.*, 1882, pp. 125 and 716, and 1895, p. 704) ; FLINT'S **Celluloid** (*Amer. Journ. Anat.*, i, 1902, p. 270) ; HUBER'S (*ibid.*, vi, 1907, p. 393) ; KRASSUSKAJA'S **Photoxylin** (*Anat.*, Heft. 2, xiii, 1904, p. 521).

552. Natural Injections (Robin, *Traité,* p. 6). To preserve these throw the organs into a liquid composed of 10 parts of tincture of perchloride of iron and 100 parts of water.

Retterer and Zenker use solution of Müller, see *Journ. Anat. Phys.*, 1894, p. 336, and *Arch. Path. Anat.*, 1894, p. 147.

553. Starch Masses. See " Guides for Vertebrate Dissection," Kingsley, New York, 1907.

CHAPTER XXV

MACERATION, DIGESTION AND CORROSION

MACERATION

554. Methods of Dissociation. It is sometimes necessary, in order to obtain a complete knowledge of the forms of the elements of a tissue, that the elements be artificially separated from their place in the tissue and separately studied after they have been isolated both from neighbouring elements and from any interstitial cement substances that may be present in the tissue. Simple teasing with needles is often insufficient, as the cement-substances are frequently tougher than the elements themselves, so that the latter are torn and destroyed in the process. In this case recourse must be had to maceration, by which is meant prolonged soaking (generally for days rather than hours) in media which have the property of dissolving, or at least softening, the cement substances or the elements of the tissue that it is not wished to study, whilst preserving the forms of those it is desired to isolate. When this softening has been effected, the isolation is completed by teasing, or by agitation with liquid in a test-tube, or by the method of tapping, which last gives in many cases (many epithelia, for instance) results which could not be attained in any other way. The macerated tissue is placed on a slide and covered with a thin glass cover supported at the corners on four little feet made of pellets of soft wax. By tapping the cover with a needle it is now gradually pressed down, whilst at the same time the cells of the tissue are segregated by the repeated shocks. When the segregation has proceeded far enough, mounting medium may be added and the mount closed.

A good material for making *wax feet* is obtained (VOSSELER, *Zeit. wiss. Mik.*, vii, 1891, p. 461) by melting white wax and stirring into it one-half to two-thirds of Venice turpentine.

The most desirable macerating media are those which, whilst dissolving intercellular substances, do not attack the cells themselves. Those which contain *colloids* have been found to give the best results in this respect. Iodised serum is an example.

555. Iodised Serum (Chap. XXI). The manner of employing it for maceration is as follows: A piece of tissue smaller than a pea must be taken, and placed in 4 or 5 c.c. of weakly iodised serum in a well-closed vessel. After one day's soaking the maceration is generally sufficient, and the preparation may be

completed by teasing or pressing out, as indicated last §; if not, the soaking must be continued, fresh iodine being added as often as the serum becomes pale by the absorption of the iodine by the tissues. By taking this precaution the maceration may be prolonged for several weeks.

This method is intended to be applied to the preparation of *fresh* tissues, the iodine playing the part of a fixing agent with regard to protoplasm, which it slightly hardens.

556. Iodide of Potassium (ARNOLD, *Arch. mik. Anat.*, lii, 1898, pp. 135 and 763). Ten c.c. of 10 per cent. aqueous sol. of potassium iodide with 5 to 10 drops of a similar solution, containing also 5 per cent. of iodine.

557. Alcohol. RANVIER employs one-third alcohol (1 part of 90 per cent. alcohol to 2 parts of water). Epithelia will macerate well in this in twenty-four hours. It macerates more rapidly than iodised serum.

Other strengths of alcohol may be used, either stronger (equal parts of alcohol and water) or weaker (¼ alcohol, for isolation of the nerve-fibres of the retina, for instance—THIN).

558. Salt Solution. Ten per cent. solution of sodium chloride is a valuable macerating medium. Weaker strengths, down to 0·6 per cent., are also used.

559. MOLESCHOTT and PISO BORME'S **Sodium Chloride and Alcohol** (MOLESCHOTT'S *Untersuchungen zur Naturlehre*, xi, pp. 99—107; RANVIER, *Traité*, p. 242). Ten per cent. solution of sodium chloride, 5 volumes; absolute alcohol, 1 volume.

For vibratile epithelium RANVIER finds the mixture inferior to one-third alcohol.

560. Sodium Chloride and Formaldehyde. GAGE recommends the addition of 2 parts of formalin to 1000 parts of normal salt solution (quoted from FISH, *Proc. Amer. Mic. Soc.*, xvii, 1895, p. 328).

561. Caustic Potash, Caustic Soda. These solutions should be employed *strong*, 35 to 50 per cent. (MOLESCHOTT); so employed they do not greatly alter the forms of cells, whilst weak solutions destroy all the elements. (Weak solutions may, however, be employed for dissociating the cells of epidermis, hairs, and nails.) The strong solutions may be employed by simply treating the tissues with them on the slide. To make permanent preparations, the alkali should be neutralised by adding acetic acid, which forms with caustic potash, acetate of potash, which constitutes a mounting medium (see BEHRENS, KOSSEL, and SCHIEFFERDECKER, *Das Mikroskop*, i, 1889, p. 156). See also GAGE, *Proc. Amer. Soc. of Microscopists*, 1889, p. 35.

562. Baryta-water, Lime-water (FOL, *Lehrb.*, p. 110). Baryta-water will macerate nerve, muscle, and connective tissue in a few hours, lime-water in a few days.

563. Sulphocyanides of Ammonium and Potassium (STIRLING, *Journ. Anat. and Phys.*, xvii, 1883, p. 208). Ten per cent. solution of either of these salts, for epithelium. Macerate small pieces for twenty-four to forty-eight hours.

SOULIER (*Travaux de l'Inst. Zool. de Montpellier*, Nouv. Sér., 2, 1891, p. 171) has found that STIRLING's solution greatly deteriorates cellular elements, but that good results are obtained by *combining it with a fixing agent.* The best results were obtained with a 2 per cent. solution of sulphocyanide combined with liquid of RIPART and PETIT; good ones, by combining liquid of RIPART and PETIT with artificial serum of KRONECKER instead of sulphocyanide, or with pepsin, eau de Javelle, 10 per cent. sulphate of soda, or 1·5 per cent. solution of caustic soda; also by combining solutions of chloride of sodium, or solutions of caustic potash or soda, with any of the usual fixing agents.

564. LANDOIS's **Solution** (*Arch. mikr. Anat.*, 1885, p. 445).

Saturated sol. of neutral chromate of ammonia	5 parts.
Saturated sol. of phosphate of potash	5 ,,
Saturated sol. of sulphate of soda	5 ,,
Distilled water	100 ,,

Small pieces of tissue are macerated for one to three, or even four to five days, in the liquid, then brought for twenty-four hours into ammonia carmine diluted with 1 volume of the macerating liquid.

GIERKE particularly recommends this liquid for all sorts of macerations, but especially for the central nervous system, for which he finds it superior to all other agents. It is also recommended for the same purpose by NANSEN (v. *Zeit. wiss. Mik.*, v, 1888, p. 242).

565. Bichromate of Potash. 0·2 per cent.

EISIG (*Fauna u. Flora Golf. Neapel*, 16 Monog., 1887, p. 297) macerates Capitellidæ in 0·5 to 1 per cent. solution for months or years, a little thymol being added against mould.

Müller's Solution, diluted to same strength, or combined with saliva, has also been used.

BROCK (for nervous system of Mollusca, *Intern. Monatssch. Anat.*, i, 1884, p. 349) takes equal parts of 10 per cent. solution of bichromate of potash and visceral fluid of the animal.

566. Permanganate of Potash is recommended, either alone or combined with alum, as the best dissociating agent for the fibres of the cornea (ROLLETT, *Stricker's Handbuch*, p. 1108). We have found it, for some objects, very energetic.

567. Chromic Acid. Generally employed of a strength of about 0·02 per cent. Specially useful for nerve tissues and smooth muscle. Twenty-four hours' maceration will suffice for nerve tissue. About 10 c.c. of the solution should be taken for a cube of 5 millimetres of the tissue (RANVIER).

568. Osmic and Acetic Acid (the HERTWIGS, *Das Nervensystem u. die Sinnesorgane der Medusen*, Leipzig, 1878, and *Jen. Zeitschr.*, xiii, 1879, p. 457).

0·05 per cent. osmic acid	. . .	1 part.
0·2 ,, acetic acid	. . .	1 ,,

Medusæ are to be treated with this mixture for two or three minutes, according to size, and then washed in repeated changes of 0·1 per cent. acetic acid until all traces of free osmic acid are removed; they then remain for a day in 0·1 per cent. acetic acid, are washed in water, stained in BEALE'S carmine, and preserved in glycerin.

For *Actiniæ* the osmic acid is taken weaker, 0·04 per cent.; both the solutions are made with sea water; and the washing out is done with 0·2 per cent. acetic acid. If the maceration is complete, stain with picro-carmine; if not, with BEALE'S carmine.

569. MÖBIUS'S **Media** (*Morph. Jahrb.*, xii, 1887, p. 174).

1. One part of sea water with 4 to 6 parts of 0·4 per cent. solution of bichromate of potash.

2. 0·25 per cent. chromic acid, 0·1 per cent. osmic acid, 0·1 per cent. acetic acid, dissolved in sea water. For Lamellibranchiata. Macerate for several days.

570. Nitric Acid. Most useful for the maceration of muscle. The strength used is 20 per cent. After twenty-four hours' maceration in this, isolated muscle-fibres may generally be obtained by shaking the tissue with water in a test-tube. Preparations may afterwards be washed with water and put up in strong solution of alum, in which they may be preserved for a long time (HOPKINS, *Proc. Amer. Soc. of Microscopists*, 1890, p. 165).

Maceration is greatly aided by heat, and at a temperature of 40° to 50° C. may be sufficiently complete in an hour (GAGE).

A mixture of equal parts of nitric acid, glycerin and water is recommended by MARCACCI (*Arch. Ital. Biol.*, iv, 1883, p. 293) for smooth muscle.

571. Nitric Acid and Chlorate of Potash (KÜHNE, *Ueber die peripherischen Endorgane*, etc., 1862; RANVIER, *Traité*, p. 79). Chlorate of potash is mixed, in a watch-glass, with four times its volume of nitric acid. A piece of muscle is buried in the mixture for half an hour, and then agitated with water in a test-tube, by which means it entirely breaks up into isolated fibres.

572. Nitric and Acetic Acid (APÁTHY, *Zeit. wiss. Mik.*, x, 1898, p. 49). 3 volumes glacial acetic acid, 3 of nitric acid, and 20 each of water, glycerin, and absolute alcohol. Macerate leeches for twenty-four hours, and bring them into 70 per cent. alcohol, in which they swell; then after twenty-four hours, 50 per cent. glycerin, changed till the acid is removed.

573. **Hydrochloric Acid.** Königstein (*Sitzb. Akad. Wien*, lxxi, 1875) takes (for gold-impregnated corneæ) equal parts of the concentrated acid glycerin, and water; Freud (*ibid.*, lxxviii, 1879, p. 102, for nerve-impregnations), 10 parts of acid, 7 of water, 3 of glycerin; and Schuberg and Schröder (*Zeit. wiss. Zool.*, lxxvi, 1904, p. 516) take (for fresh muscles of Hirudinea) hydrochloric acid of 5 per cent.

574. Béla Haller's **Mixture** (*Morphol. Jahrb.*, xi, p. 321). One part glacial acetic acid, 1 part glycerin, 2 parts water. For the central nervous system of Mollusca a maceration of thirty to forty minutes may be sufficient.

575. **Sulphuric Acid** (Ranvier, *Traité*, p. 76). Macerate for twenty-four hours in 30 grm. of water, to which are added 4 to 5 drops of concentrated sulphuric acid. Agitate. For nasal mucosa, crystalline, retina, etc.

Odenius found very dilute sulphuric acid to be the best reagent for the study of nerve endings in tactile hairs. He macerated hair-follicles for from eight to fourteen days in a solution of from 3 to 4 grm. of " English sulphuric acid " to the ounce of water.

Hot concentrated sulphuric acid serves to dissociate horny epidermal structures (horn, hair, nails).

576. **Oxalic Acid.** Maceration for many days in concentrated solution of oxalic acid has been found useful in the study of nerve-endings.

577. Schiefferdecker's **Methyl Mixture** (for the retina) (*Arch. mik. Anat.*, xxviii, 1886, p. 305). Ten parts of glycerin, 1 part of methyl alcohol, and 20 parts of distilled water. Macerate for several days (perfectly fresh tissue).

578. Gage's **Picric Alcohol** (*Proc. Amer. Soc. of Microscopists*, 1890, p. 120). Ninety-five per cent. alcohol, 250 parts; water, 750; picric acid, 1. Recommended especially for epithelia and muscle. A few hours suffice.

579. **Chloral Hydrate.** In not too strong solution, from 2 to 5 per cent. for instance, chloral hydrate is a mild macerating agent that admirably preserves delicate elements. Lavdowsky (*Arch. mik. Anat.*, 1876, p. 359) recommends it greatly for salivary glands, Hickson (*Quart. Journ. Mic. Sci.*, 1885, p. 244) for the retina of Arthropods.

580. **Lysol** (Reinke, *Anat. Anz.*, viii, 1892, p. 582). Ten per cent. solution in distilled water or in water with alcohol and glycerin. Spermatozoa of the rat or cortical cells of hairs are said to be resolved into fibrils in a few minutes, epithelial cells of salamandra to be dissociated instantaneously.

DIGESTION

581. **Digestion** is maceration in organic juices, which by dissolving out some of the constituents of tissues earlier than others serves to isolate those which resist. The chief liquids employed

are gastric juice (or pepsin) and pancreatic juice (pancreatin or trypsin).

Pepsin is best employed in acidified solution, pancreatin in alkaline.

The most favourable temperature for digestion is about 40° C.

Pepsin digests albuminoids, collagen substance and mucin more or less readily, elastin more slowly. Nuclein is either not dissolved or very slowly. Keratin, neurokeratin, chitin, fat and carbohydrates are not attacked.

Pancreatin (trypsin) digests albuminoids, nuclein, mucin, and elastic tissue; whilst collagen substance, reticular tissue, chitin, horny substances, fat and carbohydrates are not attacked.

Tissues for digestion should be fresh, or fixed with alcohol, not with chromic acid or other salts of the heavy metals.

582. Pepsin (BEALE'S, *Archives of Medicine*, i, 1858, pp. 296—316). The mucus expressed from the stomach glands of the pig is rapidly dried on glass plates, powdered, and kept in stoppered bottles. Eight-tenths of a grain will dissolve 100 gr. of coagulated white of egg.

To prepare the digestion fluid, the powder is dissolved in distilled water, and the solution filtered. Or the powder may be dissolved in glycerin. The tissues to be digested may be kept for some hours in the liquid at a temperature of 100° F. (37° C.).

BRÜCKE'S (from CARNOY'S *Biologie cellulaire*, p. 94).

Glycerinated extract of pig's stomach .	1 volume.
0·2 per cent. solution of HCl . .	3 volumes.
Thymol, a few crystals.	

BICKFALVI'S (*Centralb. med. Wiss.*, 1883, p. 838). One grm. of dried stomachal mucosa is mixed with 20 c.c. of 0·5 per cent. hydrochloric acid, and put into an incubator for three or four hours, then filtered. Macerate for not more than half an hour to an hour.

KUSKOW'S (*Arch. mik. Anat.*, xxx, p. 32). One part of pepsin dissolved in 200 parts of 3 per cent. solution of oxalic acid. The solution should be freshly prepared, and the objects (sections of hardened Ligamentum Nuchæ) remain in it at the ordinary temperature for ten to forty minutes.

583. Pancreatin. SCHIEFFERDECKER'S (*Zeit. wiss. Mik.*, iii, 1886, p. 483). A saturated solution of the "Pankreatinum siccum," prepared by Dr. Witte, Rostock, is made in distilled water, cold, and filtered. Pieces of tissue (epidermis) are macerated in it for three to four hours at about body temperature.

KÜHNE'S (*Unters. a. d. Phys. Inst. Univ. Heidelberg*, i, 2, 1877, p. 219).—Very complicated.

See also GEDOELST, *La Cellule*, iii, 1887, p. 117, and v, 1889, p. 126; MAAS, *Festschr. Kupffer*, 1899, p. 211, and HOEHL, *Arch. Anat. Phys.*,

Anat. Abth., 1897, p. 136 ($\frac{1}{5}$ to $\frac{2}{5}$ per cent. solution of Mall's or Merck's pancreatin, with 0·3 per cent. of carbonate of soda ; for demonstrating adenoid tissue in paraffin sections).

CORROSION

584. Corrosion is the operation of destroying the soft parts that surround hard parts that it is desired to study—in short, a means of cleansing hard parts for microscopic study. It has been applied to the removal of surrounding tissue from injected vessels or cavities. For this, see ALTMANN's Method (*Arch. mik. Anat.*, 1879, p. 471, or *previous editions*) ; also REJSEK (*Bibliogr. Anat.*, iv, 1897, p. 229) ; BRÜHL (*Anat. Anz.*, xiv, 1898, p. 418) ; DENKER (*Anat. Hefte.*, 1900, p. 300) ; THOMA and FROMHERZ (*Arch. Entwickelungsmech*, vii, 1898, p. 678) ; PEABODY (*Zoo. Bull.* Boston, 1897, p. 164). The following sections relate chiefly to the cleansing of native hard parts.

585. Caustic Potash, Caustic Soda, Nitric Acid. Boiling, or long soaking in a strong solution of either of these is an efficient means of removing soft parts from skeletal structures (appendages of Arthropods, spicules of sponges, etc.).

586. Eau de Javelle (Hypochlorite of Potash) (NOLL, *Zool. Anzeig.*, cxxii, 1882, p. 528). A piece of sponge, or similar object, is brought on to a slide and treated with a few drops of eau de Javelle, in which it remains until all soft parts are dissolved. (With thin pieces this happens in twenty to thirty minutes.) The preparation is then cautiously treated with acetic acid, which removes all precipitates that may have formed, dehydrated, and mounted in balsam.

The process is applicable to calcareous structures.

587. Eau de Labarraque (Hypochlorite of Soda) may be used in the same way as eau de Javelle. Looss (*Zool. Anzeig*, 1885, p. 333) finds that either of these solutions will completely dissolve chitin in a short time with the aid of heat. For this purpose the commercial solution should be taken concentrated and boiling.

If solutions diluted with 4 to 6 volumes of water be taken, and chitinous structures be macerated in them for twenty-four hours or more, according to size, the chitin is not dissolved, but becomes transparent, soft and permeable to staining fluids, aqueous as well as alcoholic. The most delicate structures, such as nerve-endings, are stated not to be injured by the treatment. The method is applicable to Nematodes and their ova, and also to the removal of the albumen from ova of Amphibia, etc.

588. Diaphanol. See § 1184. This is an important new fluid.

CHAPTER XXVI

DECALCIFICATION DESILICIFICATION AND BLEACHING

DECALCIFICATION

589. Decalcification. In order to obtain the best results, it is important to employ only material that has been *duly fixed and hardened*. Lee thought it well not to put too much confidence in reagents that are said to have the property of hardening and decalcifying fresh material at the same time.

It is generally well also to employ fluids that contain substances having a shrinking action on tissues, so as to neutralise the swelling frequently brought about by the decalcifying acids. Large quantities of liquid should be employed.

After decalcification the excess of acid should be carefully removed by washing, not in water, which favours swelling, but in some liquid that has rather a shrinking action, *e.g.* alum solution. Lastly, the tissues should be neutralised by treatment with carbonate of lime, or a salt of lithium or sodium or the like.

Rousseau (*Zeit. wiss. Mik.*, xiv, 1897, p. 207) imbeds fixed material in celloidin, brings it into 85 per cent. alcohol, decalcifies in a very acid mixture (15 to 40 per cent. of nitric acid in alcohol) washes out the acid in alcohol containing precipitated carbonate of lime, then cuts sections. This for Porifera, corals, Echinoderms, etc. Tissues are said to be well preserved.

This process has been applied to the study of the temporal bone of Mammals by Stein (*Anat. Anz.*, xvii, 1900, p. 318).

Similarly Bödecker (*Zeit. wiss. Mik.*, xii, p. 190 ; xxv, p. 21 ; xxvi, p. 206 ; and xxviii, p. 158), in a complicated way, *adding* the acid (6 to 10 per cent.) to the *thin* celloidin solution taken for imbedding.

590. Decalcification of Bone. We take the following from Busch : *Arch. mik. Anat.*, xiv, 1877, p. 481 ; see also Haug, in *Zeit. wiss. Mik.*, viii, 1891, p. 1 ; and Schaffer, *ibid.*, xix, 1903, pp. 308 and 441, and his paper in the *Enzyk. mik. Technik.*

The most widely used, though not the best, agent for decalcification is *hydrochloric* acid. Its action is rapid, even when very dilute, but causes serious swelling of the tissues. To remedy this, chromic acid or alcohol may be added to it. Or a 3 per cent. solution of the acid may be taken and have dissolved in it 10 to 15 per cent. of common salt. Or (Waldeyer) to a $\frac{1}{1000}$ per

cent. solution of *chloride of palladium* may be added $\frac{1}{10}$ of its volume of HCl.

Chromic acid is also much used, but has a very weak decalcifying action and a strong shrinking action on tissues. For this reason it should never be used in solutions of more than 1 per cent. strength, and for delicate structures much lower strengths must be taken.

Phosphoric acid has been recommended for young bones.

Acetic, lactic and *pyroligneous acids* have considerable decalcifying power, but cause great swelling. *Picrid acid* has a very slow action, and is only suitable for very small structures.

591. C. E. Jenkin's **Decalcifying and Dehydrating Fixative** (*Journ. Path. Bact.*, vol. xxiv, 1921).

Hydrochloric acid	4
Glacial acetic acid	3
Chloroform	10
Water	10
Absolute alcohol	73

Immerse tissue in 100 times its volume of the solution. The formula is based on Carnoy's fluid, and avoids the yellow colour given by the nitric acid agents, and gives a very good stain with hæmatoxylin. Wash out in absolute alcohol, several changes.

This fluid acts rapidly, a piece of human rib softening in forty-eight hours.

592. **Nitric Acid** (Busch, *loc. cit.*). To all other agents Busch prefers nitric acid, which causes no swelling and acts most efficaciously.

One volume of chemically pure nitric acid of sp. gr. 1·25 is diluted with 10 volumes water. It may be used of this strength for very large and tough bones; for young bones it may be diluted down to 1 per cent.

Fresh bones are first laid for three days in 95 per cent. alcohol; they are then placed in the nitric acid, *which is changed daily*, for eight or ten days. They must be *removed as soon as* the decalcification is complete, or else they will become stained yellow. When removed they are washed for one or two hours in running water and placed in 95 per cent. alcohol. This is changed after a few days for fresh alcohol.

Young and fœtal bones may be placed in the first instance in a mixture containing 1 per cent. bichromate of potash and $\frac{1}{10}$ per cent. chromic acid, and decalcified with nitric acid of 1 to 2 per cent., to which may be added a small quantity of chromic acid ($\frac{1}{10}$ per cent.) or bichromate of potash (1 per cent.). By putting them afterwards into alcohol a green stain is obtained.

593. **Nitric Acid** (Schaffer, *Zeit. wiss. Mik.*, xix, 1903, p. 460). Schaffer also finds nitric acid the best reagent. It should be taken pure; the addition of formol, alcohol, or the like, slows the reaction. The best strength is from 3 to 5 per cent. Objects must not be washed out directly with water, and washing in salt solution, alcohol, phloroglucin, or formol, is not sufficient to prevent swelling. Alum in 5 per cent. solution is good, but not necessary. Material should be well fixed and imbedded in celloidin (§ 182); harden in alcohol; remove the alcohol with water; put for twelve to twenty-four hours (large specimens longer) into nitric acid of 3 to 5 per cent., then into a 5 per cent. solution of sulphate of lithium or sodium, to be changed once in the

course of twelve to twenty-four hours; running water, forty-eight hours; alcohol.

594. Nitric Acid and Alcohol. Three per cent. of nitric acid in 70 per cent. alcohol. MAYER has long used 5 per cent. acid in 90 per cent. alcohol. Soak specimens for several days or weeks. Pure nitric acid, even if weak, readily exercises a gelatinising action on bone; whilst the addition of alcohol (or of *alum*) counteracts this action (FISH, *Ref. Handb. Med. Sci.*, Supp., p. 425).

THOMA (*Zeit. wiss. Mik.*, viii, 2, 1891, p. 191) takes 5 volumes of 95 per cent. alcohol and 1 volume pure concentrated nitric acid. Leave bones in this mixture, changing the liquid every two or three days, until thoroughly decalcified, which should happen, even with large bones, in two or three weeks. Wash out until every trace of acid is removed (*i.e.*, for some days after no acid reaction is obtained with litmus paper) in 95 per cent. alcohol containing an excess of precipitated carbonate of lime. This may take eight to fourteen days, after which the tissues will stain well and may be treated as desired.

595. Nitric Acid and Formol. SCHRIDDE (*Hœmatol. Techn.*, Jena, 1910, p. 21) decalcifies material fixed in formol or formol-Müller in a mixture of 1 part of formol, 1 of nitric acid, and 9 of water.

596. Nitric Acid and Alum (GAGE, quoted from FISH, § 594). A saturated aqueous solution of alum is diluted with an equal volume of water, and to each 100 c.c. of the dilute solution is added 5 c.c. of strong nitric acid. Change every two or three days, until the decalcification is complete. For teeth this is said to be, perhaps, a better decalcifier than the alcohol mixture.

597. Sulphurous Acid (ZIEGLER, *Festschr. f. Kupffer*, 1889, p. 51). A saturated solution in water. Wash out for twenty-four hours. Acts rapidly and preserves well. Best used after fixation with formol.

598. Hydrochloric Acid (see § 590). RANVIER says that it may be taken of 50 per cent. strength, and then has a very rapid action. To counteract the swelling action of the acid, sodium chloride may be added (VON EBNER), see HAUG's paper quoted § 590. He takes either 100 c.c. cold saturated solution of sodium chloride in water, 100 c.c. water, and 4 c.c. hydrochloric acid. Preparations to be placed in this, and 1 to 2 c.c. hydrochloric acid added daily until they are soft. Or, 2·5 c.c. of hydrochloric acid, 500 of alcohol, 100 of water, and 2·5 gm. of sodium chloride. HAUG prefers the proportions of 1·0 to 5·0 of acid, 70 of alcohol, 30 of water, and 0·5 of salt.

599. Hydrochloric Acid and Chromic Acid (BAYERL, *Arch. mik. Anat.*, 1885, p. 35). Equal parts of 3 per cent. chromic acid and 1 per cent. hydrochloric acid. For ossifying cartilage. HAUG recommends equal parts of 1 per cent. hydrochloric acid and 1 per cent. chromic acid (*loc. cit.*).

600. Hydrochloric Acid and Glycerin. Glycerin, 95; hydrochloric acid, 5 (SQUIRE's *Methods and Formulæ*, p. 12).

601. Trichloracetic Acid. PARTSCH (*verh. Ges. D. Naturf. Aertze*, 1895, 2 *Theil*, 2 Hälfte, p. 26) uses a 5 per cent. aqueous solution, and NEUBERGER (*Centralb. Phys.*, xi, 1897, p. 494) a 4 per cent. one. Action energetic, preservation said to be excellent.

602. Picrid Acid should be taken saturated and changed frequently. Its action is weak, but it gives good results with small objects.

Picro-nitric or Picro-hydrochloric Acid. Action very rapid.

603. **Phosphoric Acid.** Ten to fifteen per cent. (HAUG, *loc. cit.*, in § 590). Somewhat slow, staining not good. According to SCHAFFER, § 590, it produces swelling.

604. **Lactic Acid.** Ten per cent. or more. Fairly rapid, preserves well, and may be recommended (HAUG, *loc. cit.*).

605. **Chromic Acid** is employed in strengths of from 0·1 per cent. to 2 per cent. (but see § 590), the maceration lasting two or three weeks (in the case of bone). It is better to take the acid weak at first, and increase the strength gradually. Action excessively slow.

606. **Chromic and Nitric Acid.** SEILER (FOL, *Lerb.*, p. 112) takes 70 volumes of 1 per cent. chromic acid, 3 of nitric acid, and 200 of water. The action is still excessively slow, frequently requiring months to be complete.

607. **Chromo-aceto-osmic Acid** (VAN DER STRICHT, *Arch. Biol.*, ix, 1889, p. 29; and SCHAFFER, *Zeit. wiss. Mik.*, x, 1893, p. 179). Objects to be left in it for months, the liquid being changed at first every two days, afterwards less frequently. Structure well preserved.

608. **Arsenic Acid.** Four per cent. aqueous solution, used at a temperature of 30° to 40° C. (SQUIRE'S *Methods and Formulæ, etc.*, p. 11).

609. **Phloroglucin with Acids** (ANDEER, *Centralb. med. Wiss.*, xii, xxxiii, pp. 193, 579; *Intern. Monatsschr.*, i, p. 350; HAUG, *Zeit. wiss. Mik.*, viii, 1891, p. 8; FERRERI, *ibid.*, ix, 1892, p. 236; *Bull. R. Acad. Med. di Roma*, 1892, p. 67). This is said to be the most rapid method of any. Phloroglucin by itself is not a solvent of lime salts; its function in the mixture given below is so to protect the organic elements of tissues against the action of the mineral acid that this can be used in a much more concentrated form than would be otherwise advisable.

ANDEER takes a saturated solution in warm water, and adds to it 5 to 50 per cent. of hydrochloric acid. Wash out in running water.

Other acids than hydrochloric may, of course, be taken. See HAUG, *Zeit. wiss. Mik.*, viii, 1891, p. 8, and FERRERI, *Bull. Acad. Med. Roma*, 1892, p. 67, or (for both) fifth edition.

DESILICIFICATION

610. **Hydrofluoric Acid** (MAYER, *Zool. Anz.*, 1881, p. 593). The objects are brought in alcohol into a glass vessel coated internally with paraffin. Hydrofluoric acid is then added drop by drop (taking great care to avoid the fumes, which attack mucous membranes with great energy). Small pieces of siliceous sponges will be completely desilicified in a few hours, or at most a day. The tissues do not suffer.

For sponges we find that this dangerous method can be avoided. If well imbedded, sections may be made from them without previous removal of the spicules, which appear to break off sharp before the knife.

ROUSSEAU imbeds the objects in celloidin, as described § 182, then brings the block, in a covered caoutchouc dish, for a day or two into a mixture of 50 c.c. alcohol and 20 to 30 drops of hydrofluoric acid, and washes out the acid with alcohol containing carbonate of lithia in powder.

BLEACHING

611. MAYER's **Chlorine Method** (*Mitth. Zool. Stat. Neapel*, ii, 1881, p. 8). Put into a glass tube a few crystals of chlorate of potash, add 2 or 3 drops of hydrochloric acid, and as soon as the green colour of the evolving chlorine has begun to show itself, add a few cubic centimetres of alcohol of 50 to 70 per cent. Now put the objects (which must have previously been soaked in alcohol of 70 to 90 per cent.) into the tube. They float at first, but eventually sink. They will be found bleached in from a quarter of an hour to one or two days, without the tissues having suffered. Only in obstinate cases should the liquid be warmed or more acid taken. *Sections on slides* may be bleached in this way. Instead of hydrochloric acid, nitric acid may be taken, in which case the active agent evolved is oxygen instead of chlorine.

This method serves both for removing natural pigments, such as those of the skin or of the eyes of Arthropods, and also for bleaching material that has been blackened by osmic acid, and according to renewed experiments of MAYER's, is to be preferred to the peroxide of hydrogen method.

For bleaching chitin of insects, not alcohol but water should be added to the chlorate and acid (MAYER, *Arch. Anat. Phys.*, 1874, p. 321).

See also MAYER in *Zeit. wiss. Mik.*, xxiv, 1907, p. 353 (paraffin sections exposed to the vapour of chlorine water).

GRYNFELTT and MESTREZAT (*C. R. Soc. Biol.*, lxi, 1906, p. 87) add 2 c.c. of 20 per cent. solution of chloric acid (HClO]) to 15 c.c. of alcohol and put sections (of retina) into it for several hours at 42° C.

612. **Eau de Labarraque. Eau de Javelle** (see §§ 586, 587). These are bleaching agents. For the manner of preparing a similar solution see *early editions*, or *Journ. de Microgr.*, 1887, p. 154, or *Journ. Roy. Mic., Soc.*, 1887, p. 518. Of course, the method cannot be used for bleaching soft parts which it is desired to preserve.

613. **Peroxide of Hydrogen (Oxygenated Water)** (POUCHET's method, M. DUVAL, *Précis*, etc., p. 234). Macerate in glycerin, to which has been added a little oxygenated water, 5 to 6 drops to a watch-glass of glycerin. SOLGER (*Centralbl. med. Wiss.*, xxi, 1883, p. 177) takes a 3 per cent. solution of peroxide. FÜRST (*Morph. Arb. Schwalbe*, vi, 1896, p. 529) points out that after a time it macerates.

The method serves both for removing pigments and for bleaching osmic and chromic material.

614. **Peroxide of Magnesium** (MAYER, *Grundzüge*, p. 290). Use as chlorine, § 611. A slow but delicate method.

615. **Sulphurous Acid.** Prof. GILSON wrote that he found alcoholic solution of sulphurous anhydride (SO_2) very convenient for the rapid decoloration of *bichromate objects.* A few drops

suffice. MÖNCKEBERG and BETHE (*Arch. mik. Anat.*, liv, 1899, p. 135) obtain the acid by adding to 10 c.c. of a 2 per cent. solution of bisulphate of sodium 2 to 4 drops of concentrated hydrochloric acid. Objects are put into the freshly prepared solution for six to twelve hours.

616. Permanganate of Potash. ALFIERI (*Monitore Zool. Ital.*, viii, 1897, p. 57) bleaches celloidin sections of the choroid, etc., for eight to twenty-four hours in a 1 : 2000 solution of permanganate of potash, then washes them out for a few hours in a solution of oxalic acid of 1 : 300 strength, or weaker.

617. GRENACHER'S **Mixture for Eyes of Arthropods and other Animals** (*Abh. nat. Ges. Halle-a.-S.*, xvi ; *Zeit. wiss. Mik.*, 1885, p. 244).

Glycerin	1 part.
80 per cent. alcohol	2 parts.

Mix and add 2 to 3 per cent. of hydrochloric acid.

Pigments (*i.e.* those in question) dissolve in this fluid, and so doing form a stain which suffices in twelve to twenty-four hours for staining the nuclei of the preparation.

618. Nitric Acid. PARKER (*Bull. Mus. Comp. Zool.*, Cambridge, U.S.A., 1889, p. 173) treats sections (of eyes of scorpions) fixed to the slide with SCHÄLLIBAUM'S medium, for about a minute with a solution of up to 50 per cent. of nitric acid in alcohol, or, still better, with a 35 per cent. solution of a mixture of equal parts of nitric and hydrochloric acid in alcohol. To make the solution, the acid should be poured slowly into the alcohol (not *vice versâ*), and the mixture kept cool.

JANDER (*Zeit. wiss. Mik.*, xv, 1898, p. 163) takes for removal of pigments SEILER'S chromo-nitric acid (§ 606) ; twelve to forty-eight hours is enough for small objects.

See also under " Arthropoda."

619. Caustic Soda. RAWITZ (*Leitfaden*, p. 29) dissolves the pigment of the mantle of Lamellibranchia by means of 3 to 9 drops of officinal caustic soda solution added to 15 to 20 c.c. of 96 per cent. alcohol.

620. Diaphanol, § 1184. The newest method for decolorising chitin.

CHAPTER XXVII

CHROMATIN, ANIMAL * CHROMOSOMES, NUCLEOLI †

621. Study of Living Animal Cells. In the young larvæ of Amphibia, both Anura and Urodela, the gills and caudal "fin," and some other regions, may be studied *in situ* in the living state. The larvæ are usually narcotised with chloretone (in solutions of 1 to 3000 to 1 to 6000, depending on size and species) and fastened in a suitable upright chamber, such as Clark has figured and described (*Amer. Jour. Anat.*, xiii, 1912, p. 352), or they can be simply wrapped in moist blotting paper, or treated with curare, or the tail may be excised. It is preferable to cut through the larva close in front of the hind limbs.

In the *living animal* the epithelial cells and nuclei (in the state of repose) are so transparent as to be hardly visible in the natural state. They may, however, be brought out by curarising the larva; or, still better, by placing the curarised larva for half an hour in 1 per cent. chloride of sodium solution. Normal larvæ may be used for the study of the active state of the nucleus, but much time is saved by using curare.

Curare. Dissolve 1 part of curare in 100 parts water, and add 100 parts of glycerin. Of this mixture add from 5 to 10 drops (according to the size of the larva), or even more for large larvæ, to a watch-glassful of water. From half to one hour of immersion is necessary for curarisation. The larvæ need not be left in the solution until they become quite motionless, as soon as their movements have become slow they may be taken out and placed on a slide, wrapped in blotting-paper. If they be replaced in water they return to the normal state in eight or ten hours, and may be re-curarised several times.

Other Narcotics. Three per cent. alcohol or 3 per cent. ether or infusion of tobacco, may be used in a similar way. These reagents cause no obstruction to the processes of cell-division.

Indifferent Media. One per cent. salt solution, iodised serum, syrup, cold water (+ 1° C.), and warm water (35°—40° C.). The tail may be excised from the living animal and studied for a long time in these media (Peremeschko, *Arch. mik. Anat.*, xvi, 1879, p. 437).

For the processes of *staining* living cells see § 739.

Sandison (*Amer. Jour. Anat.*, xli, 1928, p. 447) has developed a transparent chamber which can be inserted into a rabbit's ear

* Read also § 1355 *et seq.* † By T. P.

and which allows normal living cells and tissues to be studied under the highest powers of the microscope. The original chamber has been modified and improved in various ways by CLARK and others (*Anat. Rec.*, xlvii, 1930, p. 147). More recently ABELL and CLARK (*Anat. Rec.* liii, 1932, p. 121) have developed an apparatus which makes it possible to introduce fluids into the chamber and thus to study the effects of various substances on the living cells.

BAUMGARTNER and PAYNE (*Jour. Exp. Zool.*, lix. 1931, p. 359) have devised a simple method of studying the spermatogenesis of the grasshopper in the living animal. The insect is first anæsthetised, and then the wings and the hind legs are removed at the autonomous joint, and a rectangular opening is made in the left side of the abdomen, from and including segments 2 to 4. The specimen is now placed on a glass slide, the right side down, and melted paraffin is run, from a pipette, over the antennæ, the legs and around again to the head leaving a space about twice the width of the animal along the dorsal side. The basin thus formed is filled with Ringer's solution (cold-blooded formula), and the testis is quickly pulled out into it, the yellow membrane removed and a few follicles are secured by a fine thread which is anchored to the paraffin. A coverslip is now placed over the basin and sealed with the paraffin to prevent evaporation.

While tissue cultures offer almost ideal material for studying living cells, there are several simple and satisfactory methods of preparation which will allow one to observe excised living cells unchanged for a number of hours. When the cells of a tissue are easily dissociated (*e.g.* the spermatocytes of insects) they can be mounted in normal body fluids, such as lymph, and examined by the hanging drop method. Since such fluids may undergo rapid changes on removal from the body, it is better, perhaps, to employ some " indifferent " medium for mounting. BĚLAŘ, who worked extensively with the spermatocytes of grasshoppers, recommends RINGER'S solution (cold-blooded formula) or TYRODE'S* solution (*Zeitsch. f. wiss. Biol., Abt. D*, cxviii, 1929, p. 359). The method is generally applicable to all tissues provided an isotonic Ringer's solution is used, *i.e.*, for warm-blooded animals use the corresponding formula. BĚLAŘ has called attention to a number of desiderata in the successful application of this method, (For a full account see *Methodik der wissenschaftlichen Biologie*, i, 1929, p. 641). Among these the following are to be noted :

It is essential that the distilled water used be free from the salts of the heavy metals and for this reason distillation from glass retorts and condensers is recommended. In removing tissue from the host, and in the subsequent manipulations, great care must be exercised to prevent the loss of water by evaporation. Cells may be dissociated from the tissue by teasing with clean needles, or in

 * §§ 780, 1430.

some other way, directly in the mounting medium, and then placed in a hanging drop, with a pipette, or mounted directly between a clean slide and coverslip. In either case the coverslip must be sealed with vaseline to prevent evaporation. Successful hanging drop preparations may be good for as long as sixty hours, but the simpler method gives normal cells for a maximum of twenty-four hours.

The mounting medium must be isotonic with the cells to be studied. In hypotonic media the cells swell and undergo other physical changes while in hypertonic solutions they shrink. Bělař suggests that the medium used for mounting be made slightly hypotonic (by the addition of distilled water) since some water is unavoidably lost in making preparations. The amount of dilution required is learned by experience.

The control of the hydrogen ion concentration of the mounting medium is an important element of success. This should be 7· or very close to it. A slight alkalinity is less harmful than an acid reaction. The *p*H may be controlled either by the addition of primary sodium phosphate (Bělař) or of carbon dioxide (Sakamura).

Another simple and useful method for studying living cells is to mount the excised tissue on a clean slide, in a mixture of normal body fluid and paraffin oil and then cover with a coverslip. For studying in the living state in Chironomus, the giant chromosomes which are found in the salivary glands, Bauer gives the following directions (*Zeitsch. f. Zellforsch. u. mikr. Anat.*, xxiii, 1935, p. 280): The glands are dissected out in the body fluid and then transferred to a clean slide, along with some of the body fluid, with a pipette. Several large drops of paraffin oil are now added to the tissue and the whole mass is covered with a coverslip. According to Bauer, the cells of the salivary gland will live a number of hours under these conditions. If the glands are taken from small larvæ of other Diptera, Ringer's solution can be used in place of the body fluids. For various Ringer solutions and Baker's solution see § 1430.

622. Study of Fresh and Lightly Fixed Cells. It is often desirable to determine quickly the character of a tissue, the stage of an embryo, the presence of mitotic figures of a certain stage, etc., before more elaborate techniques are employed. For this purpose the object to be studied can be placed in one of the indifferent media, mechanically dissociated, if need be, mounted and examined directly, or else stained with aceto-carmine or acidulated methyl green. The medium most generally used is Ringer's solution, or physiological salt solution, but there are a number of others, such as serum, iodised serum, aqueous humour, lymph, or amniotic fluid. Some of these seem to be somewhat toxic, but for the purpose in hand they are extremely useful.

For staining such temporary mounts, Belling's iron-aceto-carmine is highly recommended (see § 257), or 1 per cent. of methyl green in a 1 per cent. solution of acetic acid may be used.

A typical example of this kind of work is as follows: A bit of living tissue is teased out in Ringer's solution (the cold-blooded or warm-blooded formula, as the case may be), then placed on a slide, a coverslip added, and examined. If a stain is needed, as is usually the case, after placing the tissue on a slide the excess of Ringer is drawn off and aceto-carmine is added in excess. This fixative stain is allowed to act for two to ten minutes or more, if need be, a coverslip is placed over the tissue, the excess stain is withdrawn with filter paper and the cover-glass may be sealed with vaseline or with melted paraffin, or in some other way. It is often desirable to mash out the tissue after it has been stained. This is done by pressing in the coverslip with a blunt needle or with the tip of a finger. This should be done before the cover is sealed. Smears may be made of testicular and similar tissues and stained in this way, but in general crushed preparations are much better.

Or you may fix the preparation, after teasing, with vapour of osmium for half a minute to two minutes, then add a drop of methyl green, and after five minutes wash out with 1 per cent. acetic acid, and add solution of Ripart and Petit and cover.

Or you may kill and fix the cells by teasing in solution of Ripart and Petit (to which you may add a trace of osmic acid if you like), and afterwards stain with methyl green.

We have found Pictet's chloride of manganese (§ 442) useful as an examination medium. A little solution of dahlia may be added to it.

Henking (*Zeit. wiss. Mik.*, viii, 1891, p. 156) recommends a liquid composed of—

Water	80 c.c.
Glycerin	16 ,,
Formic acid	3 ,,
Osmic acid of 1 per cent.	1 ,,
Dahlia	0·04 grm.

Other fixing agents, such as picric acid or weak sublimate solution, may of course be used. Other stains, too, such as Bismarck brown, and of course other examination media than solution of Ripart may be employed.

But for general purposes either the aceto-carmine, or the methyl green osmium and Ripart's medium methods give such good results and are so easy and convenient to use that they should always be tried first in studies of this sort.

623. The "Nuclealfarbung" Method (Feulgen's Reaction). Of the various methods for the demonstration of chromatin, that of Feulgen seems to be the most precise and the most satisfactory, for general cytological purposes. It depends upon the presence, among the products resulting from a mild hydrolysis of nucleo-proteids, of an aldehyde grouping and the brilliant violet colora-

tion this gives with fuchsin-sulphurous acid. Needless to say, the validity of this test depends upon the absence of any aldehyde in the tissue, either produced by the cells themselves, or as the indirect effect of the fixative, and on the prevention of an oxidation of the fuchsin-sulphurous acid, which might cause the latter to act as a stain. FEULGEN (*Zeit. f. physiol. Chemie*, cxxxv, 1924) advises against the use of any fixatives which contain aldehydes or oxidising agents and recommends alcohol, acetic-alcohol and sublimate acetic. This last, which has been widely used, consists of a 6 per cent. corrosive sublimate solution to every 100 c.c. of which 2 c.c. of glacial acetic acid is added. But the fixatives recommended by Feulgen, unfortunately, are not very satisfactory cytological reagents for chromosomes. BAUER (*Zeit. f. Zellforsch. u. mikr. Anat.*, xv, 1932, p. 225) has shown, however, that, with the exception of picro-formol, Bouin's and Hermann's fluids, this test may be satisfactorily applied to tissues preserved with many of the common nuclear fixatives provided: (1) that the tissue is thoroughly washed after fixation and (2) that sections are subjected to hydrolysis for the proper length of time. Bauer has shown that there is a relatively short period during hydrolysis of animal tissues when the maximum staining reaction is obtained. Thus, following corrosive-acetic fixation, the stain is most intense when hydrolysis goes on for from four to eight minutes with the optimum at five minutes. After eight minutes the chromatin stains less deeply, and after twenty-nine minutes of hydrolysis it will not stain at all. The optimum time of hydrolysis for the commoner fixtives, as determined by Bauer, is indicated below:

Fixative.	Optimum time of hydrolysis.
Apáthy	5 minutes.
Bouin not recommended.	
Bouin-Duloscq	6 ,,
Bouin-Allen	22 ,,
Bouin-Allen with sublimate	14 ,,
Carnoy (3 : 1)	6 ,,
Carnoy (6 : 3 : 1)	8 ,,
Carnoy-Lebrun	6 ,,
Champy	25 ,,
Chromo-acetic	14 ,,
Flemming	16 ,,
Helly	8 ,,
Hermann not recommended.	
Petrunkevitch	3 ,,
Regaud	14 ,,
Regaud with sublimate	8 ,,
Zenker	5 ,,
Zenker with formol	5 ,,

Hydrolysis. The hydrolysing fluid is prepared by adding 82·5 c.c. of HCl (S.G. 1·19) to 1 litre of distilled water. The acid is placed in a staining jar or other suitable container, and brought

to a temperature of 60° C., a temperature which should be maintained throughout hydrolysis. A water bath will help to keep the temperature constant, and a variation of a degree or two will not materially alter the result, especially with fixatives requiring longer treatment. The slide, after hydrolysis, is dipped into some of the dilute cold acid solution and then rinsed in distilled water.

Staining should be carried out for one hour, according to Bauer, in fuchsin-sulphurous acid. This is prepared as follows: Bring 200 c.c. of distilled water to a boil, then add 1 grm. of basic fuchsin and stir well. Allow to cool to about 50° C. and then filter. Add 20 c.c. of the NHCl and when the temperature reaches about 25° C. put in 1 grm. of anhydrous sodium bisulphite. Sulphur dioxide is given off and the liquid slowly turns yellow. Allow to stand for twenty-four hours before using, and keep the bottle well stoppered and in the dark.

Washing. The stained slides should be passed through three washings containing sulphurous acid. Make up the following solution for this purpose:—

Distilled water	200 c.c.
10 per cent. anhydrous sol. sodium bisulphite	10 ,,
Dilute hydrochloric acid	10 ,,

The slides are next rinsed in distilled water, counter-stained in light green, if desired, and mounted in damar after dehydration and clearing in the usual way.

We find that by this method chromatin is stained a bright reddish-purple colour. Plasmosomes (or at least plastin) do not stain except with the counter stain. In animal cells cytoplasmic inclusions do not stain, but in plant tissue such substances in the cell walls as lignin, suberin or cutin may give a positive reaction. On the other hand, it is not certain that chromatin always reacts positively to Feulgen's test. In the young oocytes of some animals the nucleus fails to give an indication of chromatin by this method while the surrounding follicle cells show deeply stained nuclei. Such cases have been interpreted in two ways. Some hold that the chromatin undergoes changes in chemical composition and so does not form aldehydes, others think that the chromatin is physically too dispersed to be easily seen. Those interested in this phase will find much pertinent literature cited in a recent article by Gardiner (*Quart. Jour. Micro. Sci.*, lxxxvii, 1935, p. 523).

Literature. Feulgen, R., *Abderhalden, Hand. der biol. Arbeit. Abt. v. Lief.*, 213, 1055—1073; Ludford, R. J., *Proc. R.S.*, 1927; Verne, J., *Bull. d'Hist.*, 1927, 4, 110—122; Wermel, E., *Zeit. f. Zellforsch. u. mikroskop. Anat.*, 1926, 4, 227—232.

Chromatin is distinguished from albuminoids by not being soluble, as these are, in water and in weak mineral acids, such as 0·1 per cent.

hydrochloric acid. It is easily soluble in concentrated mineral acids, in alkalies, even when very dilute, and in some alkaline salts, such as carbonate of potash and biphosphate of soda. In the presence of 10 per cent. solution of sodium chloride it swells up into a gelatinous mass, or even, as frequently happens, dissolves entirely (CARNOY, *Biol. Cell.* pp. 208—9). It is only partially digestible (when *in situ* in the nucleus) in the usual laboratory digestion fluids.

The solvents of chromatin that are the most useful in practice are 1 per cent. caustic potash, fuming hydrochloric acid, or cyanide of potassium, or carbonate of potash. These last generally give better results than dilute alkalies. They may be employed in solutions of 40 to 50 per cent. strength. If it be desired to remove all the chromatin from a nucleus the reaction must be prolonged, sometimes to as much as two or three days, especially if the operation be conducted on a slide and under a cover-glass, which is the safer plan.

These operations must be performed on *fresh* cells, for hardening agents render chromatin almost insoluble in ammonia, potash, or sodic phosphate, etc. Hydrochloric acid, however, still swells and dissolves it, though with difficulty.

Chromatin resists the action of *digestive fluids* much longer than the albumens do ; so that a moderate digestion serves to free the chromosomes from any karyoplasmic granulations that may obscure them whilst at the same time it clears up the cytoplasm. UNNA (*Monatschr. prakt. Derm.*, xxxiii, 1901, p. 342) digests tissues in solutions of sodium chloride, to remove the " granoplasm."

ANIMAL CHROMOSOMES

624. General Remarks. There is no fixative known, for either animal or plant tissues, which will fix all parts of a cell equally well, and for detailed cytological studies *it is desirable, at the outset, to decide whether nuclear or cytoplasmic structures are of paramount importance.* The reason for this seems to be, in part, that nuclear fixatives must be strongly acid in order to preserve the chromatin well and give clean cut fixation images after staining. On the other hand, the cytoplasm and its inclusions seem to require, in general, fixatives which are nearer neutrality, though let it be noted that all fixatives are on the acid side of pH 7·0 (exceptions to the general rule are Benda's fixative (pH 1·2) and Champy's fluid (pH 1·4). To illustrate the effects of hydrogen ion concentration, ZIRKLE (*Protoplasma*, iv, 1928, p. 201) has shown that when bichromate fixatives are used which are on the acid side of the critical range, pH 4·2–pH 5·2, the nucleolus, chromatin, spindle fibres and spongioplasm are fixed, while nuclear lymph, mitochondria and hyaloplasm are dissolved. But on the basic side of the critical range the chromatin and spindle fibres are destroyed while the other elements persist.

YOMAHA (*Bot. Mag. Tokyo*, xxxix, 1925, p. 167) has measured the hydrogen ion concentration of most of the common fixatives and finds this to range from pH 1·1 to pH 3·0. (For a discussion of the pH see ATKINS in the last edition.)

If it were possible to expose naked chromosomes directly to the action of fixatives, we could probably develop one fluid which would preserve all types of chromosomes well, at a given phase of mitosis or meiosis. But in practice, the intervening cellular structures, which must be penetrated before the chromosomes are affected, force us to greatly modify our technique for various types of animal and plant tissue. While practically all of the nuclear fixatives have been developed empirically, and we know very little regarding either the chemistry or physics of fixation, within the past decade we have gained some insight into a few factors which affect the penetration of reagents, and the effect of hydrogen ion concentration. And a brief summary of the facts may serve to explain, to the reader, why we have in use, at the present time, a number of fixatives which contain the same ingredients and differ only slightly in the proportions of the reagents.

ZIRKLE (*Protoplasma*, iv, 1928, p. 201) has shown that the fixation image is largely determined by the reagent which first reaches the cell structure. As an example, tissue fixed in a mixture of formol and acetic acid may give the " acid " fixation image in the cortical layers, where no mitochonchia will be found, while deeper within, the mitochondria (fixed first by the formalin) show with appropriate stains. The penetration of a given reagent is dependent upon its concentration (CROZIER, *Jour. Gen. Physiol.*, v, 1922, p. 65). Different tissues may affect the concentration of a reagent in different ways, not only by the physical density of cell parts, and by simple dilution, but also by some sort of chemical union between the tissue and the reagent. In these respects no two fixative agents may be just alike. Another factor which may greatly disturb the normal fixation image is the nature of the substances which are dissolved by the fixative. Thus, Zirkle has shown that the tannic acid present in some plant tissues may affect the action of reagents to a marked degree.

Zirkle has made a number of studies on the effect of hydrogen ion concentration on fixation. Using the same ingredients but changing the *p*H of the fluids, may give the most diverse fixation images. See ZIRKLE, *Protoplasma*, x, 1934, p. 31, for literature.

In the light of the foregoing discussion it may be said that there are two cardinal points in making preparation for chromosome studies. The first is that the material to be preserved must be absolutely fresh, a condition especially important for animal tissues, where a few minutes' delay may profoundly affect the whole nuclear structure before the fixative is applied. The second is that, no matter what fixation is employed, the cells to be fixed must be brought into as close contact with the fixative as possible. One well fixed cell is, in general, more useful than thousands which are not, and it is a waste of time to preserve large hunks of tissue for chromosome study. In dense tissues, such as the testes of many vertebrates, it is rare to find well preserved chromosomes more than four or five cell layers from the surface. Other things being equal, the more finely divided the

tissue is, therefore, the more likely one is to obtain good fixation.

625. General Methods. In animals, material for chromosome studies may be found either in germinal or in somatic tissue. Germ cell divisions, especially meiotic stages, must be sought in the gonads at the proper time in the life cycle, which varies in different groups. Somatic divisions are most numerous in embryos and in larval stages. Among the higher vertebrates the amnion gives excellent chromosome figures and this tissue has been used extensively in mammals having a high chromosome number. Dividing mesenchyme cells give good figures provided the embryo has been cut up so that this tissue is well preserved. Among the invertebrates, especially in insect larvæ, the brain and the larger ganglia have been favourite tissues.

In the past, the standard procedure has been to make paraffin sections of fixed tissue and to stain these in iron hæmatoxylin, or some other nuclear dye. Some investigators have used the smear method (see §§ 645, 1126 for details) for spreading the tissue into a thin layer, after which it is fixed and stained by the usual methods. More recently, many cytologists have found that excellent chromosome preparations may be had, if organs, such as the gonads of insect larvæ, are killed and stained with aceto-carmine, and then spread out or mashed, by pressure on the coverslip until a thin layer of cells is obtained. This last method is so quick and simple that it should be thoroughly tried out before more elaborate techniques are resorted to (see §§ 257, 634 for details).

Fixatives. For animal tissues the inexperienced worker will do well to begin with Bouin's fluid or some other picro-formol-acetic combination, because these penetrate evenly and deeply, do not overfix, are cheap and easy to use. The main disadvantages of such solutions are, first, some cytoplasmic structures are not fixed well, and, certain nuclear anilin dyes do not take as well after picric acid fixation as they do after the use of osmic acid, and it is necessary to mordant with the latter if these special dyes are to be used (see § 1364). For these reasons, for a well-rounded study of cellular structures picro-formol-acetic fixation should be supplemented by material treated with Flemming's solution, or some one of the other chromo-aceto-osmic mixtures. The latter have the disadvantages, however, of not penetrating well, of overfixing if applied too long, of being expensive, and finally, of requiring a bleaching of the tissue before staining.

After one has become familiar with a particular type of tissue, it will often be profitable to vary the proportions of the ingredients a little, for experience has shown that even slight variations may have a marked effect both on the density of cell parts and many of the finer details. For example, there are numerous slight modifications of Bouin's, of Flemming's solutions, and of the

chromo-formol-osmic-acetic mixtures, which have become standard for specific types of tissues or for a given stage of mitosis or meiosis. With regard to the chromosomes it must be realised that they are complex structures which vary both in their physical and chemical state in the several phases of mitosis, and a fixative which, for example, gives very satisfactory metaphase plates may preserve the more disperse phases very poorly. In selecting the method to be followed, some thought should be given to the type of study it is desired to make. When the counting of chromosomes is a primary consideration, a certain amount of shrinkage is not detrimental and may be a positive advantage, when high numbers are dealt with. For such studies picro-formol-acetic, or chromo-aceto-osmic mixtures should be tried first, or if unavoidably dense membranes must be penetrated, such as a thin layer of chitin, then Carnoy's fluid or some one of the mercuric fixatives such as Gilson's or Petrunkevitch's is recommended. But when one wishes to study the internal structure of chromosomes, shrinkage is to be avoided, as far as possible, and certain pre-fixation treatments may be extremely valuable. In the sections following the reader will find detailed information about the various methods which are considered, by the writers, as the best for a particular type of tissue or phase of mitosis for animals and plants.

626. Hot and Cold Fixation. Nuclear fixatives are commonly employed at room temperature, but some workers advocate the use of warm or hot fluids and others believe the best results are obtained by keeping the vial of fixative (previously chilled by ice) on ice while the material is being fixed. Cold Flemming's solution is recommended by EZRA ALLEN (*Anat. Rec.*, x, 1915, p. 16), and HANCE (*Anat. Rec.*, xii, 1917, p. 371) for the preservation of mammalian chromosomes. The fixative which is generally used warm, at 37°–38° C., is Allen's modification of Bouin's fluid. When hot fixatives are used, the period of fixation is reduced. It is possible that the chilling alters the physical state of the protoplasm, allowing the fixative to penetrate before the chromosomes have clumped, and that heat accelerates the penetration of the reagents. In any event, we feel that the matter will repay investigation and that cytologists will do well to try both hot and cold methods.

627. Stains. The iron alum hæmatoxylin method of Heidenhain has always been and is now the chromosome stain *par excellence.* Most cytologists, perhaps, prefer the short method, *i.e.*, mordanting from a few minutes up to an hour or two and staining a correspondingly short time. At present, possibly the next most popular stain is gentian violet, especially the method of Newton (§ 1364) or of La Cour (§ 1364), but it should be noted that this stain will fade after certain types of fixation. Feulgen's

stain (§ 623) gives excellent results and should be more generally employed. And there are, of course, many other nuclear stains which are satisfactory when skilfully used.

628. Fixation of Mammalian Chromosomes. The material studied is generally either the testis of the adult or tissue taken from a very young embryo (the amnion gives excellent figures). In either case it is absolutely essential that fresh material be used, since even a delay of one or two minutes may cause a clumping of the chromosomes.

If the testis is to be preserved, no anæsthetic should be used. Either the animal is castrated quickly, or it can be stunned or killed by a blow on the head and the testis quickly removed. Some workers prefer to slice the testis into thin layers a millimetre or two thick and preserve these, others cut the testis into small pieces and then tease these directly in the fixing agent. Experience has indicated (see, for example, WINIWARTER and OGUMA, *Arch. d. Biol.*, xxxvi, 1926, p. 102) that it is inadvisible to place mammalian testis in physiological salt, or in Ringer's solution, in order to separate the tubules of the testis. Mammals vary greatly in the ease with which the tubules may be separated, the mouse and rat and some other rodents being the least difficult in this respect. In working with germinal tissue, due regard must be given to the breeding season of the form and only healthy and properly nourished animals should be used.

When preserving embryos, if the amnion is to be studied, this is simply exposed unbroken and the embryo is dropped into the preserving fluid. After washing, small sections of the amnion are stained with iron hæmatoxylin and mounted *in toto* in damar or balsam, after clearing with one of the vegetable oils (oil of cedar is excellent).

If one wishes to study dividing cells in mesodermal or nervous tissue (in vertebrates the former is better), it is well to chop up the embryo before preservation because good chromosome plates are not found, as a rule, more than a few cell layers from the surface of the tissue.

Three general methods have been employed for the fixation of mammalian chromosomes, all of which have given excellent figures.

H. DE WINIWARTER (*Arch. d. Biol.*, xxvii, 1912, p. 91) and some others have used Flemming's solution, in which the amount of acetic acid has been reduced. HANCE (*Anat. Rec.*, xii, 1917, p. 371) adds a little urea (about 0·5 per cent.) to Flemming's solution, which is chilled on ice. The tissue is placed in this cold fluid and kept there for about twenty-four hours. (The temperature of the fixative on ice registers about 4° to 5° C.) More recently, OGUMA and KIHARA (*Arch. d. Biol.*, xxxiii, 1923, p. 493) have fixed thin slices of human testis for one minute in Carnoy's solution (the 6 : 3 : 1 mixture), and then transferred them to strong Flemming's solution for twenty-four hours.

Painter (*Anat. Rec.*, xxvii, 1924, p. 77), and many others, have used a slight modification of Allen's modification of Bouin's fluid (10 c.c. of acetic acid is used instead of 5 c.c. in the formula given in § 115). Tissue is cut into small pieces and teased directly in the preservative with needles. Fixation lasts for $1\frac{1}{2}$ to 3 hours. The drop method is used for changing fluid, and Allen's anilin-wintergreen oil method of clearing is followed (see § 629 for details).

The Japanese cytologists have been using, with success, either the original Champy's formula, or some modification of it. (See especially Minouchi, *Jap. Jour. Zool.*, viii, 1928, p. 219, also § 632 below.) After sectioning and bleaching, sections are treated for twenty-four hours with Chura's solution (*Zeit. wiss. Mikr.*, xiii, 1925), which consists of equal parts of glacial acetic acid and a saturated aqueous solution of picric acid, in order to dissolve out the cytoplasmic inclusions and to render the chromosomes more deeply staining.

If the tubules of the testis are teased apart, it is important that these small structures be protected from injury during dehydration and imbedding. In the higher grades of alcohol, especially after osmic acid fixation, the tubules become extremely brittle and the very best fixed material may be lost. It is advisable to put the testicular fragments into a little cage of some sort during dehydration, clearing and imbedding. The common Gooch crucible is good for this purpose, or a small square of porous cloth may be folded into a bag to hold the material.

629. Precautions in Dehydrating and Clearing. It is well known that too rapid dehydration and clearing will cause shrinkage and often distortion. Material to be used for chromosome study should be dehydrated gradually, either by the drop method, or by some one of the devices which have been developed to ensure the gradual exchange and diffusion of fluids. For clearing, the least shrinkage seems to occur with some of the vegetable oils, such as bergamot, wintergreen, cedar wood or origanum, and if any of these do not mix readily with paraffin, the clearer can be washed out with benzol or xylol. The use of xylol, as a clearing agent directly from alcohol, is not recommended for delicate tissues as it causes some shrinkage.

Ezra Allen (*Anat. Rec.*, x, 1915, p. 565), following Suchannek (§ 150) uses anilin oil as a substitute for alcohols by a method developed to follow his modification of Bouin's fluid, but applicable generally to other types of fixation *except those involving the use of osmic acid.*

Following Allen's lead, Painter employs the following procedure: After fixation with Bouin-Allen, small pieces of the testis are placed on a square of cloth of porous weave, the four corners are brought together, forming a sort of bag. A pin is

stuck through the corners of the cloth and on into a cork. The cork, with the tissue hanging down beneath, is floated on the surface of any sort of cylinder, such as a graduated one of 500 or 1,000 c.c. capacity, containing 5 per cent. alcohol. Here the fixative will diffuse out of the tissue and go to the bottom of the cylinder. After a few hours to overnight, the bag of tissue is fastened to the wall of a shell vial with any ordinary paper clip, and the dilute alcohol is gradually replaced by dropping in, with a capillary syphon, 50 per cent. alcohol until a concentration of about 40 per cent. is reached within the vial. During this and the subsequent steps in dehydration, the fluid within the vial is agitated either with compressed air, or by a plunger. If the fluid which is being dropped into the vial has a lighter specific gravity than that in the vial, the former should be introduced by a small glass tube running from the syphon to the bottom of the vial. After the tissue is in 40 per cent. alcohol, a mixture of equal parts of anilin oil and 50 per cent. alcohol is dropped in until saturation for the mixture is reached. (Ordinarily, about 150 to 200 c.c. is passed through a vial of about 25 c.c. capacity during the course of three or four hours. Note: The mixture of anilin oil and alcohol may absorb enough moisture, on damp days, from the atmosphere, to cause some of the oil to be thrown out of solution and making the fluid within the vial become cloudy. The addition of a few drops of 95 per cent. alcohol will generally clear up the solution again.) Next, a mixture of anilin oil and 70 per cent. alcohol (equal parts) is added by the drop method. Then, in turn, comes pure anilin oil and oil of wintergreen, the time taken to add each one of these being about four hours. After clearing in wintergreen oil, the tissue is removed from the cloth bag, which during the steps just described has protected the tiny tubules from mechanical injury. Imbedding is by steps, some seven to ten changes being used, thus one part of paraffin to seven of wintergreen oil, etc. Painter has found it very convenient to use, during imbedding, ordinary Naples' staining jars, with Gooch crucibles which fit snugly into the open top. The paraffin and wintergreen oil tend to separate out on standing, so that each mixture must be well agitated before use. The whole imbedding process should not take over two or three hours.

630. Avian Chromosomes. The methods given above for mammals are generally applicable here. In the adult active testis the tubule walls are very thin and the contents so fluid that on rupture the germ cells tend to flow out into a solid mass, making preservation difficult. One should either cut very thin slices of the testis, or else tease bits of the testis directly in the preserving fluid. In either case, great care should be exercised in the subsequent handling of the tissue so that the tubules lying on the

surface, which becomes very brittle in the higher grades of alcohol, are not broken off and the best preserved cells lost.

For the preservation of testicular material, OGUMA (*Jour. Col. Agri.*, Hokkaido Imp. Univ, xvi, 1927, p. 203) uses Hermann's fluid (containing about 1 per cent. urea) warmed to about 40° C., and fixes for a few hours. Painter, and many others, have used Bouin-Allen followed by the anilin and wintergreen oil method of imbedding. For embryonic divisions, Hance recommends strong Flemming (with 1 per cent. urea added) chilled with ice (§ 626). Oguma prefers warm Hermann's fluid. Amniotic divisions in six to ten-day-old embryos are excellent for somatic chromosomes.

631. Reptilian Chromosomes. The methods employed for mammals will be found good. NAKAMURA (*Mem. Col. Sci.*, Kyoto Imp. Univ., Series B, lv, 1928, p. 1) has used the following modification of Champy's fluid which is highly recommended, also, by MATTHEY (*Rev. Suisse Zool.*, xxxvii, 1931, p. 117) : 2 per cent. osmic acid, 3 parts ; 1·6 per cent. chromic acid, 6 parts ; and 6 per cent. potassium bichromate, 4 parts. Tissue is fixed in this mixture for twenty-four hours, washed for the same length of time, slowly dehydrated, cleared in cedar oil, then in chloroform, and imbedded. Sections are bleached in hydrogen peroxide in 50 per cent. alcohol and then placed in Chura's fluid for twelve to twenty-four hours, to dissolve out cytoplasmic inclusions and to render the chromosomes more easily stainable.

632. Amphibian Chromosomes. Except for maturation divisions in oocytes, Bouin-Allen is excellent for germinal and somatic divisions. Recently, some Japanese cytologists, *e.g.*, MAKINO, *Jour. Fac. Sci.*, Hokkaido Imp. Univ., ii, 1932, have found that either Benda's or Flemming's solutions are excellent if the acetic acid is reduced or omitted. For the meiotic divisions in mature eggs, MAKINO (*Jour. Fac. Sci.*, Hokkaidic Imp. Univ., *Zool.*, iii, 1934, p. 117) uses a saturated solution of mercuric chloride containing about 1 per cent. of acetic acid. Eggs are fixed for ten to fifteen minutes and washed in 70 per cent. alcohol. The gelatinous envelopes should be removed from the eggs before fixation. If the eggs have been in water for a little while, this can be easily done with a pair of scissors. Freshly laid eggs, or ones taken from the oviduct, are preserved and washed first and are then placed in a 10 per cent. solution of formol This causes the membranes to become brittle, and they may now be removed with needles. After dehydration, cedar oil is used for clearing, and then the eggs are placed in a mixture of creosote and toluol (equal parts), where they are left for about thirty minutes. The creosote is washed out with toluol and the eggs are imbedded.

633. Teleost Chromosomes. IRIKI recommends Champy's fluid (*Proc. Imp. Acad.*, Tokyo, viii, 1932, p. 262). MAKINO (*Cytologia*, v, 1934, p. 155) finds that Champy's works well with spermatocyte stages, but for spermatogonial divisions Benda's fluid diluted

with equal parts of water seems better. PROKOFIEVA (*Cytologia*, v. 1934, p. 498) uses equal parts of 5 per cent. chromic acid and 50 per cent. formol as a fixative.

634. Methods for Invertebrate Chromosomes. As in all chromosome work, the key to successful fixation is bringing the unaltered living tissue into direct contact with the fixing agent. As a rule, metaphase chromosomes present no great difficulty, but for early meiotic stages, in general, and especially for ova which carry large amounts of yolk, or are surounded by dense envelopes, special methods must be employed.

In the case of small animals, such as fleas or lice, the end of the abdomen may be cut off and the viscera squeezed out on a glass slide which is quickly plunged into a jar of the fixative. For larger animals, one may open up the body cavity and pipette fixative over the viscera before the gonads or nervous tissue are separated away. It is generally better, however, to dissect out the desired tissue in normal body fluids, or in some medium like Ringer's solution (cold-blood formula) or an isotonic salt solution. (Isotonic salt solution ranges from 0·70 to 0·75 per cent. of sodium chloride). Sometimes a 2 per cent. solution of urea is used as the dissecting medium. With any of these methods great care should be taken not to allow either the tissue or the dissecting medium to undergo any evaporation. In the past the favourite fixatives have been chromo-aceto-osmic mixtures, especially Flemming's solution, and picro-formol-acetic combinations, such as Bouin's fluid. For many types of chromosome work, the iron-aceto-carmine technique is quite adequate, and in cytogenetic laboratories, it is being extensively employed.

For eggs surrounded by chitinous, or other more or less impervious, membranes, one must use fixatives with great penetrating power. One will be well repaid, however, for the time spent in removing or puncturing the envelopes, when this is possible. The fixatives generally employed are the fluids of Carnoy, either the 6 : 3 : 1, or the 1 : 1 : 1 formula, Gilson and Petrunkevitch. For the eggs of many insects, Kahle's fluid is recommended, but the egg envelopes must be punctured before its use. It is usually necessary to take special measures to ensure the easy sectioning of such material (§ 172).

635. Illustrative Examples. In this section the methods currently employed for the study of *Drosophila* chromosomes will be described in detail and will serve as a guide for the study of various types of insect tissues. Following this, will be found information about the fixatives commonly used for the study of the chromosomes of other types of invertebrates.

636. Oogonial Divisions. These are found in the ovaries of late pupæ or in adult flies up to twelve hours after hatching. The ovaries are dissected out either in Ringer's solution (cold-blood formula) or in 2 per cent. urea, or in a 0·73 per cent. solution of sodium chloride.

If *aceto-carmine* preparations are desired, the ovaries are placed on a clean slide with a pipette, the dissecting medium quickly removed (avoid any evaporation) and iron-aceto-carmine is added from one side in considerable excess. (Adding the stain in this way usually causes the ovaries to stick to the slide and makes the subsequent handling of the material easier.) The ovaries are stained until they are a dark red, which will take from ten to twenty minutes, and then the coverslip is added. A little evaporation of the stain during the process seems desirable. The excess of stain is removed with a pipette and filter paper and then the preparation is blotted with a good deal of pressure. The oocyte divisions are at the small end of the egg strings, so the ovaries should be mashed out by pressing on the coverslip with a blunt needle. The coverslip is sealed with vaseline, melted paraffin, or in some other way. The preparation is now ready for study, and if artificial light is used, it is well to filter it through a blue-green filter, or a yellow-green filter. Such a slide is best a few hours after staining and will continue useful for several days depending on climatic conditions.

If *sections* are desired, the ovaries are transferred from the dissecting medium to the fixative, using a pipette. Strong Flemming's solution is often employed, but it should not be allowed to act more than thirty minutes, less is probably better, and then the hardening should be completed in some other medium. Bridges (personal communication) uses a 1 per cent. solution of chromic acid in which the ovaries are left for a few hours. Painter transfers the ovaries to Hermann's fluid, in which they are left two or three hours (*Zeitsch. ind. Abs. Verer.*, lxii, 1932, p. 316). After fixation the ovaries are washed for four or more hours. Then they should be packed into pupa cases, which enables one to carry them through dehydrating and imbedding without trouble. (See § 157 for directions for handling small objects). The reason ovaries are left in the Flemming's solution such a short time is that longer fixation causes the cytoplasm of the cells to take on a grey tone, when iron hæmatoxylin is applied. In the experience of the writer, Bouin's fluid, or Bouin-Allen, causes the chromosomes to contract much more than they do in osmic fixatives.

If desired, *smears* may be made of ovaries in the usual way (see § 625).

637. Spermatogonial and Meiotic Stages. These must be sought in very young larvæ. A larva, reared at ordinary room temperatures, will show all stages up to spermatids, when it is four days old. Spermatogonial divisions may be found occasionally in the adult testes. Any of the standard fixatives can be used. (At this early age one cannot distinguish between male and female larvæ, but when one dissects out the gonads, the testes will be found to be considerably larger than ovaries in larvæ of the same size.)

638. Somatic Divisions. Both prophase and metaphase stages should be sought in the brain or in the large ganglia of young larvæ. Dissections can be made in Ringer's solution (cold-blood formula) or in 0·73 per cent. salt solution. Navaschin's fluid has been widely used as a fixative for such tissue, and sections should be stained in iron hæmatoxylin or in Newton's iodine gentian violet. According to the recent work of PROKOFIEVA (*Zeitsch. Zellforsch. mikr. Anat.*, xxii, 1935, p. 254) the chromo-formol fixatives of Levitsky (§ 1330) are valuable for showing the constrictions. The morphology of the X chromosome shows best after treatment with equal parts of 10 per cent. formol and 1 per cent. chromic acid. For the autosomes she uses 6 parts of 50 per cent. formol and 4 parts of 5 per cent. chromic acid.

639. Drosophila Eggs and Embryos. The first step is to remove the chorion from the freshly laid eggs. This is easily done in the following way: Several years ago Professor J. T. Patterson, at the University of Texas, found that if a little quick-drying glue is smeared on a slide and the eggs are placed on this fresh surface, when the glue is dry the chorion may be removed from the eggs by pressing against one side with a blunt needle. The de-chorionated eggs are next punctured on the convex side, slightly posterior to the middle with a very sharp needle. HUETTNER (*Jour. Morph.*, xxxvii, 1922, p. 385) found that Kahle's formol-alcohol-acetic fixative gave the best results. Others prefer Petrunkevitch's fluid. Very young ovarian eggs are well preserved with Flemming's solution, as described above, and section easily.

640. Salivary Gland Chromosomes. The general experience has been that old fat larvæ, raised on rich yeast food at a low temperature (from 16° to 20° C.), show the largest chromosomes and give the best preparations. The glands are dissected out in Ringer's solution (cold-blood formula) or in a 0·73 per cent. salt solution. Subsequent treatment depends on the type of preparation desired.

For a study of the living chromosomes the glands may be mounted on a clean slide with some of the dissecting medium and the coverslip sealed with vaseline. Care should be taken to prevent evaporation during the process. Perhaps it would be better to adapt the method recently used for the study of *Chironomus* chromosomes by BAUER (*Zeitsch. f. Zellforsch. u. mikr. Anat.*, xxxiii, 1935). Bauer dissects large larvæ in their own body fluids and with a pipette transfers the glands, along with some of the hæmolymph, to a clean slide, where several large drops of paraffin oil are added. A coverslip is now placed over the whole mass and the preparation is ready for study. It will remain fresh up to twenty-four hours.

641. For temporary aceto-carmine preparations. PAINTER (*Genetics*, xix, 1934, p. 175) uses the following method: After

dissection in Ringer's solution, the glands are placed on a clean slide, the excess fluid removed, and iron-aceto-carmine is added from one side. After a minute or two the stain is removed and replaced by fresh fluid, thus ensuring that there will be no dilution due to the dissecting medium. The stain is allowed to act for ten to twenty minutes, depending on the sample of stain, room temperature and, perhaps, the condition of the larvæ. When the glands are a deep red in colour the coverslip is added (it may be placed over the glands when they are first mounted, if desired), and then the excess stain is drained off with a pipette and filter paper. Next the preparation is blotted with filter paper, using a good deal of pressure, both to crush the glands and to remove more stain. It is essential that the coverslip is not allowed to move during the blotting, for this will break the chromosomes and otherwise badly distort them. The nuclei are usually freed from the surrounding cytoplasm, when the glands are crushed, but the walls generally remain intact. The next step is to rupture the nuclear walls so that the chromosomes can spread out. This is best done under a wide field binocular by pressing on the coverslip with a blunt needle directly above a nucleus. Do not allow the coverslip to move about. Any excess stain is now removed from the preparation and the coverslip is sealed with melted paraffin, vaseline, or in some other way. Such a slide will last for several days or longer, if it is kept cool. The reader should note that the success of the aceto-carmine method depends upon having a good stain with just the right amount of iron. Read carefully the directions given in § 257.

642.* For making permanent aceto-carmine slides several very good methods are in current use among *Drosophila* workers, and it is too soon to say which should be standard practice. The preliminary steps are described in the preceding paragraph; further treatment consists of dehydrating the preparation and mounting in euparal, Venetian turpentine or balsam or damar The writer has found that the methods used by Bridges and others (see Drosophila Information Service No. 6) give unusually clear preparations. The larvæ are reared at a low temperature. and given food very rich in yeast. When they crawl up on the paper, or the walls of the bottle, preparatory to pupating, they are removed and the glands are dissected out in cold Ringer's solution (or in a 0·73 per cent. salt solution) and transferred to a dish of plain (without iron) aceto-carmine which has been chilled with ice, where they are left for twenty minutes to several hours. The glands are then mounted as described above, but instead of sealing the coverslip the slide is placed in a dish containing alcohol fumes (any convenient dish may be lined with filter paper and saturated with 95 per cent. alcohol). The slides are allowed to " season " in alcohol fumes up to twenty-four hours. An hour is said to be

* See also p. 277.

sufficient, though a longer time works well. Next the slides are placed in a jar of 95 per cent. alcohol where they may be left overnight or longer. Here a few of the coverslips will loosen and may even come off. If they do not, they are pried off with a needle ground to a spade-like edge and mounted directly in euparal, further dehydration being unnecessary. With a little care the coverslip may be put back into the same position it had when it was pried off.

When the coverslip is pried off, as described above, some of the tissue will stick to the slide, some to the cover and some will come loose and be lost in the mounting process. Bauer (communicated by letter) has found that the following treatment of the slides and coverslips will remedy this trouble: Before use, the slides are given a thin coating of egg albumin, which is allowed to dry. The under side of the coverslip is coated with a thin layer of oil (run your finger tips through your hair and then rub the underside of the cover with them). This will keep the chromosomes from sticking to the coverslip when the nuclei are crushed.

If one wishes to employ Feulgen's stain, from the 95 per cent. alcohol the slides are brought through the lower grades to water and then fixed a few minutes with Bouin-Allen (presumably other fixatives will do also), washed in running water, after which they are treated and stained in the usual way (§ 623).

643. Other Examples. For orthopteran chromosomes CAROTHERS (*Jour. Morph.*, xxviii, 1916) recommends a modification of Bouin's fluid (see § 115), which is currently widely used. MINOUCHI (*Zeitsch f. Zellforsch. u. mikr. Anat.*, xx, 1934, p. 709) finds the best fixation, after the use of diluted Flemming's solution from which all acetic acid is omitted. Dilute two or three times with distilled water.

For lepidopteran chromosomes Seiler has used Carnoy's 6 : 3 : 1 mixture for testes and PETRUNKEVITCH's fluid for eggs (*Arch. Verer. Social u. Rasshygiene*, i, 1925, p. 63). GOLDSCHMIDT (*Arch. f. Ent. d. Org.*, cxxvi, 1932, p. 591) recommends the use of Bouin-Duboscq for such material.

For crustacean testes, Bouin-Allen has been used with good results. More recently, NIIYAMA (*Jour. Fac. Sci.*, Hokkaido Imp. Univ., iii. 1934, p. 4) finds that Champy's fluid diluted with an equal part of water works well with a fresh-water crayfish. For the edible crab, one should use sea water for dilution (*ibid.*, iv, 1935, p. 59).

For arachnida, SOKOLOW (*Zeitsch. f. Zellforsch u. mikr. Anat.*, xxi, 1934, p. 42) recommends that the testes be dissected out from the liver in a 0·75 per cent. solution of sodium chloride. Aceto-carmine preparations give excellent figures, but if sections are required, such fixatives as Flemming's, Champy's or Navaschin's work well.

For annelid chromosomes Huth reports that for meiotic and first cleavage stages in eggs, Bouin or Bouin-Allen give excellent results. For earlier stages of the ovary, and for testes, Flemming's or Navaschin's fluids are much better (*Zeitsch. f. Zellforsch. u. mikr. Anat.*, xx, 1933, p. 309).

For trematode eggs, Minouchi fixes (Polystomum intergerrimum) for thirty to forty minutes in Carnoy's 1 : 1 : 1 mixture and then replaces

this slowly with Flemming, where the eggs are left for twenty-four hours (*Zeitsch. f. Zellforsch. u. mikr. Anat.*, xxiv, 1936, p. 85).

For nematodes, *Ascaris megalocephala*, for example, oogonia, oocytes and fertilisation stages preserve well with Bouin-Duboscq, Flemming, Zenker, or Petrunkevitch. Cleavage stages require such mixtures as Carnoy's (1 : 1 : 1) Carnoy-Bebrun, or acetic-alcohol.

644. Mounting Sections between Coverslips. Agar (*Quart. Jour. Micr. Sci.*, 1911) has devised a method for mounting preparations between coverslips in order that they can be observed on both sides. Use one larger coverslip as if it were a slide. Carriers may be made by stamping out a square in stiff cardboard, or thin metal. See also, C. Cépède, *C. R. Soc. Biol.*, clv, 1913.

645. Smear Preparations of Gonads. In some cases smear preparations of testes especially may provide useful evidence in a research on chromosomes. One may be fortunate enough to find nuclei at the prophase or metaphase of mitosis, with all the chromosomes spread out so as to be counted with ease. In many cases, to study early stages in synapsis for which very rapid penetration is essential, smear preparations are a *sine qua non*.

Remove the testes ; if it is large take a fragment by a pair of forceps and quickly smear along the length of a dry slide several times, so as to cover as much of the middle part of the slide as possible. If the cells are likely to stick, fix immediately by pouring on some Flemming, Bouin, or Petrunkewitsch. Set aside for a few minutes, wash off in water, upgrade from 30 per cent. alcohol, and leave overnight in 90 per cent. alcohol. Bring back to water, stain in iron hæmatoxylin, thionin, or gentian violet, etc. See also Goodrich's iodine-Bouin method (under " Protozoa "). Note that smears may be fixed in steam, acetic, osmic, formalin vapour, or stained and fixed simultaneously in Leishmann, acetic Bismarck brown (§ 346), aceto-carmine (§ 257), or such mixtures. Smears of very liquid testes, like those of Lepidoptera, are liable to be washed away if fixative is added too soon. It is probably best to kill the cells in some toxic vapour, then allow them to dry a little and then fix in a liquid.

Foot and Strobell (*Arch. f. Zellf.*, Bd. xii, 1914) recommended the following : Place testes in drop of acidulated (acetic) " water " on end of slide, and with fine needle (No. IX.) cut from it the area at stage required (previously ascertained by examination of sections). Push this area by point of needle to middle of slide and break up the tissue by gently tapping with the needle (never roughly spread as recommended by some workers). This should be done under dissecting microscope.

See also Kernschwarz, and " Iron Carmine," § 258. For Bataillon and Koehler's borax-methylen-blue see *Comptes Rendus*, cxvii, 1893, p. 521.

NUCLEOLI AND CENTROSOMES

646. The word nucleolus is generally used to mean any large rounded stainable body in the nucleus, but cytologists recognise that at least two types of substances are included under this name, chromatin and so-called plastin. Chromatin nucleoli are made up of chromatin, principally at least, and may represent whole chromosomes, such as the sex chromosomes in the auxocytes of

many insects, or short sections of chromosomes which remain condensed (heteropycnotic) during the interphases of mitosis, or possibly, are formed in other ways. Such structures stain as chromatin with the common nuclear (basic) dyes, and give a positive reaction to Feulgen's stain following the fixatives ordinarily used for the preservation of chromosomes. The "plastin," of which plasmosomes are wholly or mostly composed, is acidophil and does not stain with Feulgen's method. Some nucleoli are compound in character and contain both chromatin and plastin.

Although our knowledge of nucleoli is still incomplete, the following facts seem well established with regard to them: (1) While typical chromatin nucleoli stain with basic dyes and plasmosomes with acid stains, under certain conditions not well understood, *i.e.,* during spermateleosis of the sperm head, chromatin may take acid dyes. It is also well known that plasmosomes may be made to react differently to a specific dye depending both on the nature of the fixative agents and the *p*H. For example, in plant cells fixed with Bouin's fluid, the plasmosome stains intensely with iron hæmatoxylin and holds this dye after long extraction. If Navaschin's fluid is used for fixation, however, it shows little affinity for this stain. (2) It has been shown repeatedly that plasmosomes may arise from, or in intimate association with, a definite region of a specific chromosome. In some instances the connection between the two persists. This being so, we might expect that bodies made up of plastin might show traces of chromatin, and *vice versâ.* (3) There is no reason, *a priori*, to suppose that plasmosomes are made up of only one sort of material. It is possible that diverse acidophilous substances appear as plasmosomes.

Nucleoli are commonly studied by three diverse methods. One involves reactions to dyes alone, the second consists of impregnating with silver or osmium, and the third involves differences in solubility of nuclear components.

Nucleoli are comparatively easily preserved and any of the standard chromosome fixatives may be used. Chromo-aceto-osmic mixtures are the best, perhaps, because of the ease with which some double stains follow this fixation. To distinguish between the chromatin and plastin the most satisfactory method we have at present is Feulgen's stain followed by some counter-stain, such as light green. If this method is to be followed, select one of the fixatives which lends itself to this test. See § 623 for further details. Other tests are given in a paragraph below. For staining nucleoli the principal stains which are commonly employed are: safranin and methyl green, iron hæmatoxylin (after strong Flemming fixation), Auerbach's methyl green and acid fuchsin (this does not work well after fixation

with Bouin's fluid), Pappenheim's stain and Mann's methyl-blue-eosin method. LUDFORD (*Jour. Roy. Mic. Soc.*, 1922, p. 113) obtained his best preparations after fixing with corrosive acetic and staining with Mann's stain. In the oocytes of molluscs he found that both the oxyphil and basophil nucleoli gave a negative reaction with the Feulgen test (*Proc. R. S. B.*, cii, 1928).

In studying nucleoli one should use more than one set of stains and also other methods outlined below.

The impregnation methods of Cajal, or of Da Fano, may prove very useful.

There are a number of reagents which react differentially upon chromatin and plastin, enabling one to separate these two chemically. One of the simplest, and according to Saguchi the most satisfactory, is to fix the tissue in absolute alcohol and after washing place it in a 0·1 per cent. solution of potassium hydroxide. Even after two to four minutes most of the chromatin will be dissolved while the plasmosome will not be affected. After longer treatment, the tissue can be imbedded and sections stained in the ordinary way. (See SAGUCHI, *Zytologische Studien*, Hefte vii, 1934. Other methods are also described in this paper and pertinent literature cited.)

Zirkle has developed several fixatives designed to aid in distinguishing nuclear components. (See especially, *Cytologia*, ii, 1931, p. 85, for formulæ and literature.) The following solution is said to fix plastin and destroy chromatin : Potassium bichromate 1·25 to 1·5 grm., ammonium bichromate 1·25 to 1·5 grm., sodium chromate 1·0 to 0·5 grm., and aq. dest., 100 c.c.

647. Centrosomes. These structures are relatively easy to preserve. Among the fixatives generally employed are Bouin's and Flemming's fluids and sublimate combinations. Tissue should be thoroughly washed after fixation and very thin paraffin sections are advisable. The best method of staining, so far developed, is iron hæmatoxylin. Thin sections should be mordanted in fresh 2·5 per cent. ferric alum for twelve to twenty-four hours and stained in hæmatoxylin for several days. Counter-staining with acid fuchsin or light green is often helpful. Mallory's phosphotungstic acid hæmatoxylin is recommended by STURDIVANT (*Jour. Morph.*, xlv, 1933, p. 435) as the best progressive hæmatoxylin stain. The practice of Heidenhain of staining tissue first in Bordeaux R., and then in iron hæmatoxylin, seems no longer to be followed.

* 642. ZIRKLE (*Science*, May, 1937) recommends for permanent mounting the following :—Acetic acid (glacial) 50 c.c., water 50 c.c., glycerin 1 c.c., gelatin (powdered) 10 grm., dextrose 4 grm., $FeCl_3$ 0·05 grm., carmine to saturation. Dissolve gelatin in water, add other components, boil, filter. Use as ordinary aceto-carmine, or dilute in various proportions with Belling's aceto-carmine.

CHAPTER XXVIII

FATTY SUBSTANCES *

648. Nomenclature.

Each of the general terms in use has more than one meaning. The following outline of current usage is based chiefly on Sperry's discussion of nomenclature (*A Textbook of Biochemistry*, ed. B. Harrow and C. P. Sherwin. Saunders, Philadelphia and London, 1935, p. 109).

Each of the words, *fat*, *lipoid*, *lipide*, *lipid*, *lipin*, may denote either (1) fatty substances in general or (2) one or more groups of fatty substances defined according to chemical properties. *Fat* in the restricted sense denotes glyceryl esters of fatty acids, compounds referred to also as *neutral fats* or *true fats*. *Lipoid* in the restricted sense may denote either (1) fatty substances exclusive of neutral fats or (2) fatty substances containing nitrogen. Used with reference to the results of histological examination of tissue sections *lipoid* usually denotes sudanophil substances either shown or assumed to contain cholesterol. Biochemists are agreed that the word *lipoid* should be abandoned in favour of *lipide*, *lipid*, or *lipin*, but the relative claims of these words are unsettled and no definition of any of them is universally accepted.

The only general terms used in this chapter are "fat" and "fatty substances"; each of these terms covers fatty acids and soaps, glyceryl esters of fatty acids, waxes, sterols and their esters, phosphatides and cerebrosides.

649. Scope of Histological Methods. The fat that can be studied by histological methods forms a variable fraction of the total fat content of any tissue. This fraction may be termed *visible* to distinguish it from the *invisible* fraction whose existence can be established only by chemical analysis.

Many methods have been devised for the study of visible fat, but common experience and critical examination have shown that few are really valuable. The outlook on fat staining has been revolutionised during the past ten years. Of the many studies that have contributed to this change, those of Kaufmann and Lehmann (1926 *a* and *b*, 1928–29, 1929, 1932) and Lison (1933 *a* and *b*, 1934, 1935 *a* and *b*, Lison and Dagnelie, 1935) deserve special mention. Lison's work is summarized in his recent book (1936). His first paper (1933 *a*) is a critical review covering almost the whole of the literature. The reader is referred to the following literature :

* By W. W. K. and R. W.

Arndt, H. J., *Verh. deuts. path. Ges.*, xx, 1925, p. 143; Brunswik, H., *Z. wiss. Mikrosk.*, xxxix, 1922, p. 316; Daddi, L., *Arch. ital. Biol.*, xxvi, 1896, p. 143; Froboese, C. and Spröhnle, G., *Z. mikr.-anat. Forsch.*, xiv, 1928, p. 13; Gross, W., *Z. wiss. Mikrosk.*, xlvii, 1930, p. 64; Kaufmann, C. and Lehmann, E., *Zbl. allg. Path.*, xxxvii, 1926*a*, p. 145; *Arch. path. Anat.*, cclxi, 1926*b*, p. 623; *Ibid.*, cclxx, 1928–29, p. 360; *Z. mikr.-anat. Forsch.*, xvi, 1929, p. 586; *Arch. path. Anat.*, cclxxxiii, 1932, p. 190; Kay, W. W. and Whitehead, R., *J. Path. Bact.*, xxxix, 1934, p. 449; *Ibid.*, xli, 1935, p. 303; Kimmelstiel, P., *Zbl. allg. Path.*, xxxvi, 1925, p. 491; *Krankheitsforsch.*, v, 1927, p. 403; Larson, H. W., *J. Lab. Clin. Med.*, xviii, 1933, p. 848; Laux, F. J., *Zbl. allg. Path.*, xxxviii, 1926, p. 581; Leulier, A. and Noël, R., *Bull. Histol. appliq.*, iii, 1926, p. 316; Leulier, A. and Revol, L., *ibid.*, vii, 1930, p. 241; Lison, L., *ibid.*, x, 1933*a*, p. 237; *ibid.*, x, 1933*b*, p. 292; *C. R. Soc. Biol.*, cxv, 1934, p. 202; *Arch. Biol.* (Liége), xlvi, 1935*a*, p. 599; *Bull. Histol. appliq.*, xii, 1935*b*, p. 279; *Histochimie animale*, Gauthier-Villars, Paris, 1936 (Part II, Section III, pp. 189–224); Lison, L. and Dagnelie, J., *Bull. Histol. appliq.*, xii, 1935, p. 85; Mayer, R. M., *Krankheitsforsch.*, vi, 1928, p. 48; Romeis, B., *Arch. path. Anat.*, cclxiv, 1927, p. 301; *Z. mikr.-anat. Forsch.*, xvi, 1929, p. 525; Romieu, M., *C. R. Soc. Biol.*, xcii, 1925*a*, p. 787; *C. R. Assoc. Anat.*, xx, 1925*b*, p. 345; Schultz, A., *Zbl. allg. Path.*, xxxv, 1924–25, p. 314; *Verh. deuts. path. Ges.*, xx, 1925, p. 120; Schultz, A. and Löhr, G., *Zbl. allg. Path.*, xxxvi, 1925, p. 529; Sehrt, E., *Histologie und Chemie der Lipoide der weissen Blutzellen*, Thieme, Leipzig, 1927, p. 24 (cited by Froboese and Spröhnle, 1928); Steinle, J. V. and Kahlenberg, L., *J. Biol. Chem.*, lxvii, 1926, p. 425; Whitehead, R., *J. Path. Bact.*, xli, 1935, p. 305; Yamasaki, K., *Fukuoka-Ikwadaigaku-Zasshi*, xxiv, 1931, p. 79.

VARIOUS METHODS AND THEIR RELATIVE MERITS

650. Stains for Fatty Substances in General. The most widely used method for fatty substances in general is staining with Sudan or Scharlach. Other substances proposed include cyanine, alkanna, chlorophyll, indophenol, extracts of capsicum berries and of carrot; none of these is in general use and according to Lison (1934) all, with the possible exception of indophenol, are inferior to Sudan and Scharlach. After studying the fat-staining properties of many dyes, Lison (1934) recommended the following, which he stated were specific for fatty substances: blue B.Z.L. (Ciba), Sudan red B, and Sudan black B (both I. G. Farbenindustrie).

651. Examination of Vacuoles in Paraffin Sections. Since dehydrating and clearing agents are fat-solvents, fatty substances are represented in paraffin sections as a rule merely by vacuoles. It is unjustifiable to regard the study of such vacuoles as a method for fatty substances for the following reasons: (1) it is irrational to study fatty substances indirectly when direct methods are available, (2) not all vacuoles represent fatty substances, (3) it is impossible to appreciate in vacuolated paraffin sections details that are obvious in sections containing fatty substances, and (4) no test can be applied to vacuoles.

652. Polariscopic Examination. The following account is based on Lison's (1933 *a*) conclusions.

When examined in polarised light between crossed nicols fatty substances may be :—

(1) *Isotropic* (*non-luminous*). This result may be due to any kind of fatty substance in liquid form : neutral fats and fatty acids when liquid are never anisotropic; cholesteryl esters, phosphatides, and cerebrosides may be, but are not always, either because they are above their clearing points, *i.e.* the maximal temperatures at which they can exist as liquid crystals, or because the formation of liquid crystals is inhibited in some way.

(2) *Anisotropic* (*luminous*).

(*a*) *Not showing a Black Cross of Polarisation.* This result may be due to any of the substances named under (1) when in solid crystalline form.

(*b*) *Showing a Black Cross of Polarisation.* This result may be due to any of the substances named under (1) except neutral fats and fatty acids and indicates the presence of liquid crystals.

It is impossible polariscopically to distinguish between neutral fats and fatty acids or between cholesteryl esters, phosphatides and cerebrosides. Polariscopic examination is thus not a method for determining the composition of fatty substances, although it is sometimes useful for studying their morphological distribution. It is necessary for identifying sterol-digitonide in applications of the Windaus test for free sterols.

653. Methods Formerly Supposed to be of Histochemical Value. By means of osmic acid it was formerly considered possible to distinguish neutral fats from cholesteryl esters, phosphatides, and cerebrosides. Osmic acid, however, does not react with all fatty substances, and does react with many that are non-fatty; it is therefore not a reagent even for identifying fatty substances in general, and has no histochemical value whatever (Lison, 1933 *a*). After detailed critical examination (1926 *a* and *b*, 1928–29, 1932) Kaufmann and Lehmann concluded that none of the following methods was specific : Ciaccio's for "lipoids," Smith (Nile blue), Fischler, and Smith-Dietrich. Lison has since found (1935 *a*, and, with details, *b*) that commercial Nile blue is impure, containing, in addition to true Nile blue, a substance that he terms Nile red. The pink colour of fatty substances stained with commercial Nile blue is due exclusively to this Nile red, which when pure stains fat in the same way as Sudan; no conclusion regarding the composition of fatty substances is possible from the results with either Nile red or pure Nile blue. These conclusions supersede those Lison gives in his book (1936, p. 203). Lison (1933 *a*) proposed the retention of the Smith-Dietrich method as being specific for phosphatides and cerebrosides provided that cholesterol and its esters could be excluded. He assumed that this condition

was fulfilled if histological applications of the Liebermann-Burchardt test gave constantly negative results. The assumption is, however, unjustifiable, as Lison himself had recognised (1933 *a*, p. 272).

It is impossible by any stain to establish chemical distinctions between various kinds of fatty substances ; the only methods that can now be recognised as differential are not stains but chemical tests, and those that are generally accepted are exclusively for sterols and their esters.

654. Methods for Sterols and their Esters. The techniques of Schultz (1925), Romieu (1925 *a* and *b*), and Yamasaki (1931) are applications of the Liebermann-Burchardt cholesterol test. Applied to animal tissues they are specific for cholesterol whether in the free or ester form. The only technique in general use is that of Schultz.

Larson (1933) proposed a test based on the work of Steinle and Kahlenberg (1926). It depends on the formation of an addition product of cholesterol with antimony pentachloride. In view of the similar reactions given by antimony pentachloride with other fatty substances (Steinle and Kahlenberg, 1926) it is doubtful whether Larson's test is specific.

The techniques of Brunswik (1922), Leulier and Revol (1930, superseding Leulier and Noël, 1926), and Lison (1936) are applications of the Windaus digitonin method and are specific for free sterols. Although sound in principle these techniques need further investigation.

655. Value of Methods Discussed. 1. *Staining with dyes of the Sudan type :* the best method for demonstrating fatty substances in general.

2. *Examination of vacuoles in paraffin sections :* irrational and misleading.

3. *Polariscopic examination :* of limited histochemical and morphological value only.

4. *Osmication, Ciaccio's method for "lipoids," Smith (Nile blue), Fischler, and Smith-Dietrich methods :* these have no histochemical value.

5. *Techniques based on the Liebermann-Burchardt test :* when applied to animal tissues, specific for cholesterol whether in the free or ester form : the only technique in general use is that of Schultz.

6. *Techniques based on the Windaus digitonin test for free sterols :* specific for free cholesterol in animal tissues, but need further investigation.

7. *Other methods :* either need further study or have no advantage over those named.

656. Methods Recommended. The methods recommended are

1. Sudan staining for fatty substances in general.

2. The Schultz test for cholesterol whether in the free or ester form.

THE SUDAN METHOD

657. Principle. Sudan colours fat by dissolving in it and the process of staining consists in the passage of dye from one solvent (usually alcohol) to another (fat) in which it is more soluble. Little is known of the various factors that may influence this passage.

658. Composition of Commercial Sudans. The dye sold under the name Sudan III is reputed to be benzene-azo-benzene-azo-β-naphthol. The dyes sold under the names Sudan IV, scarlet or scarlet R (and corresponding words in foreign languages), and Fettponceau, are reputed to be toluene-azo-toluene-azo-β-naphthol. The powders supplied under these various names differ in both their chemical and staining properties. Sehrt (1927) noticed that different samples of the same brand varied in quality. Romeis (1927) stated that commercial Sudan III contained three dyestuffs, which he termed Sudan red, Sudan orange, and Sudan yellow; their chemical nature was not determined. Kay and Whitehead (1934) studied three brands of Sudan III, referred to as A, B and C. A furnished excellent fat stains; later investigation suggests that it was true Sudan IV. B, a widely used German brand, gave fair results; it was a mixture of dyes only a small proportion of which resembled true Sudan III. C was useless as a fat stain; it was probably true Sudan III. Kay and Whitehead (1934) found one brand of scarlet R useless for fat staining owing to its ready solubility in water. According to common experience alcoholic solutions of Sudan III are brown or brownish-red; Daddi (1896), who introduced Sudan III as a fat stain, described its alcoholic solution as scarlet, a word more nearly descriptive of alcoholic solutions of true Sudan IV. True Sudan IV is undoubtedly superior to Sudan III because the red colour it imparts to fatty substances contrasts more sharply with the background of a section than the yellow, orange, or brown of Sudan III.

659. Solvents for Sudan. The usual solvent is 70 per cent. ethyl alcohol. This has the disadvantage of extracting some of the fat, and various other solvents have therefore been tried. Romeis (1927, 1929) devised a technique in which the dye was dissolved in 40 per cent. alcohol. Objections were raised by Froboese and Spröhnle (1928), but Kaufmann and Lehmann (1929) concluded that the Romeis technique was the best available. Neither the technique nor the interesting discussion it provoked can, however, be regarded as more than suggestive because the chemical nature of the dyestuff used by Romeis was unknown.

According to Gross (1930) the loss of fat inevitable with alcoholic

solutions is obviated by the use of diacetin, which dissolves the dye but not the fat.

660. Technique of Staining. The dye recommended is toluene-azo-toluene-azo-β-naphthol. A brand of this has been specially prepared by the British Drug Houses Ltd., and is sold as Sudan IV (B.D.H., No. 555722).

The following solutions are needed (Kay and Whitehead, 1935) : (A) *Stock solution of dye.* Add 2 g. Sudan IV powder to 1 litre of absolute ethyl alcohol at room temperature, boil gently till all the powder has dissolved, and allow to cool and settle. (B) 45 *per cent. alcohol.* Mix 4 volumes of absolute ethyl alcohol with 5 volumes of distilled water. (C) *Staining solution : saturated solution of Sudan IV in* 70 *per cent. alcohol.* To 7 volumes of (A) add slowly, with shaking, 9 volumes of (B). Mix thoroughly, allow to stand for one hour, and filter.

Formol-fixed frozen sections are placed successively in :—(1) 50 per cent. alcohol, five minutes ; (2) solution (C), thirty minutes at 37° C. ; (3) 50 per cent. alcohol, a few seconds ; (4) distilled water, a few minutes at least ; (5) filtered hæmalum ; (6) alkaline tap water, a few minutes at least ; and (7) mounted in glycerin jelly.

661. Notes on Technique. (1) The alcohol used to prepare solution (A) must be absolute. Any loss by evaporation while the dye is being dissolved must be made good by adding more alcohol while the solution is hot. (2) (A) when hot is transparent; while cooling it becomes slightly turbid. A small deposit of excess dye forms on the sides and bottom of the bottle and the cold solution is transparent. (3) Within a few minutes after (A) and (B) have been mixed, the mixture, (C), becomes turbid. If (C) is used immediately, even though filtered, a precipitate may form rapidly on the sections. This is avoided or delayed for some weeks by allowing (C) to stand for one hour before filtering. (4) (A) keeps for at least six months without deteriorating. (C) rapidly deteriorates and should be discarded within about four hours after being mixed ; it may, however, be used repeatedly during this period. (5) Sections should be stained on the day after cutting. Sections stained on the day of cutting tend to stick to the glass rod used for manipulation. If staining is delayed for more than a few days after the end of fixation the results are often unsatisfactory, probably owing to the crystallisation of the fatty substances. The finished preparations deteriorate in the course of weeks or months. (6) Precautions to prevent evaporation of (C) are unnecessary. Hollow glass stoppers from wide-mouth 2-oz. bottles make convenient staining vessels. (7) If sections overlap, the covered portion is stained less strongly than the uncovered. Sections may be unevenly stained even though they were not overlapped. The risk of uneven staining is minimised

if the sections are turned over after fifteen minutes in (C). (8) For an indication of the results obtainable see the coloured plate published by Whitehead (1935).

THE SCHULTZ CHOLESTEROL TEST

662. Principle. The Schultz cholesterol test is an application to histological sections of the Liebermann-Burchardt cholesterol test (Schultz, 1924–25; Schultz and Löhr, 1925; Schultz, 1925). The colour reaction is said to be due, not to cholesterol itself, but to some product of its oxidation, termed oxycholesterol (Windaus, cited by Schultz, 1925). The reaction was originally obtained after exposing sections to strong light, but treatment with metallic salts, especially iron alum, was found to be equally effective. When a mixture of glacial acetic and sulphuric acids is added to a treated section, the development of a blue-green colour indicates that cholesterol was present in the section before it was treated.

663. Specificity. Kimmelstiel at first doubted (1925), but later admitted (1927), the specificity of the reaction. The conclusions of Schultz were supported by Arndt (1925), Laux (1926) and Mayer (1928), and confirmed completely by Kaufmann and Lehmann (1926 *a*, *b*). They applied the test to 109 substances and found that the result was never positive in the absence of cholesterol although it might be negative even when cholesterol was present. Cholesteryl stearate, for example, gave a negative result, and mixtures containing it gave positive results only if cholesterol itself was among the ingredients. Since cholesterol by itself gives a positive result (after preliminary treatment) its presence would explain the positive result with such a mixture. The results with the following mixtures (Kaufmann and Lehmann, 1926 *b*) are instructive. Cholesterol-lecithin +; cholesterol-lecithin-glycerin —; cholesterol-lecithin-glycerin-stearic acid +. Glycerin thus inhibited the reaction, but its effect was annulled if stearic acid was present. These examples suggest that a negative result in histological sections may be due to many causes.

In animal tissues a positive result is specific for cholesterol, whether in the free or ester form, because no other sterol is present in significant amounts. In plant tissues substances other than sterols and their esters might give a positive result.

664. Technique. The following solutions are needed:

(A) 10 *per cent. solution of iron alum.* Dissolve at room temperature 100 g. violet iron alum, $(NH_4)_2SO_4 \cdot Fe_2(SO_4)_3 \cdot 24H_2O$, in distilled water and make up volume to 1 litre. (B) 2·5 *per cent. solution of iron alum.* Dilute 1 volume of (A) to four times its volume with distilled water. (C) *Acid mixture.* Add slowly, with external cooling, concentrated sulphuric acid to an equal volume of glacial acetic acid. Formol-fixed frozen sections are: (1) Placed

in (B) for three days at 37° C.; (2) rinsed in distilled water; (3) mounted on a slide; (4) blotted dry with filter paper; (5) treated with a few drops of (C); and (6) covered with a cover-glass.

665. Notes on Technique. (1) The acid mixture should be made by putting the glacial acetic acid in a flask and slowly adding the sulphuric acid with careful shaking, the flask being kept cool by immersion in cold water. (2) Both acids must be of "analytical reagent" standard, and the sulphuric at least 98 per cent. H_2SO_4. After the acid mixture has been added to a section a few bubbles usually appear, but do not interfere with the appreciation of colour. If the acids are impure, however, bubbles may appear in large numbers and cause serious difficulty. The acid mixture is hygroscopic and the bottle containing it must therefore be kept stoppered when the mixture is not in use. If due care is exercised the acid shows no sign of deterioration even after a year's regular use. (3) If the iron alum solution is heated during preparation basic iron sulphate is precipitated; this cannot be redissolved. Both 10 per cent. and 2·5 per cent. solutions keep well. The 2·5 per cent. solution is discarded after being used once. The object of preparing a 10 per cent. solution is to reduce labour when large volumes of the 2·5 per cent. solution are being used. (4) Gelatine embedding, advised by Schultz (1925), is unnecessary. A film of egg albumen on the slide keeps the section flat during the performance of the test. The test is applicable also to urinary deposits and sputa, which are dried on a slide, fixed in formol, and then placed in the iron alum solution. (5) At the start of a test a dry glass rod should be inserted in the acid bottle and returned to it immediately after it has been used to add acid to a section. If the rod is left exposed to the air the film of acid on the rod will be rapidly diluted by atmospheric moisture. At the end of a test the acid bottle should be stoppered and the glass rod washed in water and dried. (6) The blue-green colour appears within a few seconds after the acid mixture has been added and becomes stronger during the next few minutes. It becomes dirty brown within half an hour and sections must therefore be examined without delay. Permanent preparations are unobtainable. Fatty substances giving a negative result appear brown. (7) The operator may check his reagents and technique by testing sections of a suprarenal gland from a guinea-pig or rabbit killed in good health; the fatty substances in the cortex give a strongly positive result, even in sections that have been kept in formol for months. (8) Whenever a section gives a negative result, further sections should always be examined to exclude technical errors. Sections of a tissue known to be Schultz-positive should be tested at the same time.

CHAPTER XXIX

MICROCHEMICAL TESTS FOR CERTAIN SUBSTANCES OTHER THAN FATS AND CHROMATIN

666. In this chapter are some methods for glycogen, iron, copper, calcium and lead. It should be stressed that microchemistry of tissues carried out by staining or otherwise treating sections or smears is full of possible pitfalls. It needs a knowledge of both organic and inorganic chemistry often of a highly specialised nature; only those methods which are fairly straightforward and which offer a good chance of success are given here. Many of these methods used in modern histo-chemistry have been worked out for mammalian tissues and when applied to invertebrate or plant tissues are of more doubtful value. Even such apparently straightforward methods as those for glycogen are not easy and unless special care is taken the results obtained will not be of value. The reader is advised to consult Dr. Lison's recent work "Histochimie animale: Méthodes et Problèmes," Gauthier-Villars, Paris, 1936, which is devoted entirely to this subject, and which discusses the value of each test in a critical manner. The success of a number of newer biochemical colour tests applied to sections and smears made in this laboratory has not been impressive.

Elsewhere will be found tests for chromatin, § 623; fats, § 650; oxidation centres, § 888; and for materials in plant tissues, § 1337, chitin, etc., § 1189.

GLYCOGEN

667. Glycogen is a carbohydrate which occurs in many cells, both glandular and genital: it is found in both inter- and intracellular positions, in the form of small areas of flocculent appearance. For its study in a tissue or organ two methods should be used:—(1) An iodine technique; and (2) that of Best's carmine. The specificity of the latter method has been questioned, and both techniques must be used for comparison.

668. Iodine Method. Fix tissue in Carnoy, or alcohol absolute, 4 parts; acetic acid glacial, 1 part; or in absolute alcohol; or in alcoholic fixatives not containing alcohol lower than a strength of 90 per cent. It is better if the tissue is cut small. Fix for one hour, then transfer for twenty-four hours or longer, in two changes of absolute alcohol; then xylol and paraffin wax. Fix sections

to slide with a mixture of 50 per cent. alcohol with a few drops of glycerin and albumen, using the alcohol as you would water; drain the slides dry. Remove wax in xylol, bring to 70 per cent. alcohol. Stain sections in Ehrlich's hæmatoxylin for five or ten minutes. Blue in tap water substitute, § 1429 *bis*. Pass to a 2 per cent. solution of potassium iodide saturated with iodine (a Lugol solution); leave five minutes; pour away, wipe around slide, and dehydrate in absolute alcohol saturated in iodine. Clear in oleum origanum cretici for about ten minutes. Mount in origanum balsam. Such preparations should keep for years without fading much. We have some slides of human placenta which after twenty years still show the glycogen.

This is described by Lison as "Méthode de Langhans, variante de Carleton." It was, however, used in this form by Jenkinson and S. G. Scott previously. We do not know the exact origin of this variation. It was published in this text-book in the 8th edition.

It is not specific, since amyloid and certain proteid granules take the colour as well. Spit on one slide and use it as a control, § 670.

669. Ehrlich's Gum Iodine. Dissolve 50 grm. of gum arabic in 100 c.c. of distilled water. The gum should be put in a net bag or the solution filtered. Add 1 grm. of iodine dissolved in a little water in which 3 grm. of potassium iodide have been dissolved previously. Fix material in alcohol; for Protozoa, smear, fix in alcohol and add gum glycerin, *apply coverslip after about one quarter of an hour*. Glycogen and paraglycogen brown. The preparations are semi-permanent.

670. BEST'S **Carmine Stain.** Material is fixed as for the iodine method and may be imbedded in celloidin. If paraffin sections are used the slide must be placed in 1 per cent. celloidin overnight, drained and allowed to dry partly, and then plunged into chloroform and absolute alcohol (equal parts), then treated as for celloidin sections. Transfer through alcohols 90 per cent. and 70 per cent. to water. Stain in Ehrlich's or iron hæmatoxylin as usual, but differentiate in acid alcohol. Then proceed to BEST'S carmine stain (*Zeit. f. mikros.*, Bd. xxiii). Make up this stock carmine solution :—

Carmine	2 grm.
Potass. carbonate	1 grm.
Potass. chloride	5 grm.
Aq. dest.	60 c.c.

Boil gently for a few minutes; cool.

Add strong liq. ammon. 20 c.c. Keep this solution in a well-stoppered bottle in a cupboard. It may go bad in a month during summer.

Wash sections in distilled water after staining in hæmatoxylin. Stain in following solution :—

Stock carmine solution	2 parts
Liq. ammon. fort.	3 ,,
Methyl alcohol (pure)	3 ,,

for five minutes.

Differentiate in—

Absolute alcohol	80 parts.
Methyl alcohol	40 ,,
Aq. dest.	100 ,,

till no more red comes out (three to five minutes).

Wash in 80 per cent. alcohol, absolute and clove oil, xylol and xylol balsam ; nuclei and cytoplasm, blue, glycogen red.

It is a good plan when working on glycogen to prepare triplicate slides, one for iodine stain, one for Best ; the other slide is brought down to water and spat upon and set aside : the glycogen is dissolved by the diastase of the saliva, the latter is washed off in water and the slide stained as usual for Best's carmine. Comparison between the first slide and this one will assist in properly identifying glycogen (procedure of the late Dr. B. R. G. Russell, *Imperial Cancer Research Laboratory*).

One generally succeeds at first trial with such material as the liver of a rabbit, but with invertebrate materials, especially from paraffin sections, even though soaked in 1 per cent. celloidin, the results are often disappointing. This can be overcome by practice and by slight modification in the time used for differentiation. For delicate material it seems best to work with celloidin sections.

671. **Zieglwallner's Alcoholic Flemming for Glycogen and Fat.** Neither the iodine nor Best's carmine method preserves fat as well as glycogen. Zieglwallner has worked out the following method for preserving both fat and glycogen. Fix small pieces of tissue in this mixture for twenty-four to forty-eight hours :—

1 per cent. chromic acid in 80 per cent. alcohol	15·0
2 per cent. OsO_4 in water	4·0
Acetic acid	1·0

In 100 c.c. of this mixture there would be 60 per cent. alcohol.

If a corrosive sublimate fixation is necessary use this mixture in the same way :—

Concentrated aq. sol. corrosive sublimate	20·0
2 per cent. OsO_4 in water	20·0
Acetic acid	10·0
Alcohol absolute	50·0

In washing out, a little iodine will be necessary. Transfer the pieces of tissue to 70 per cent. alcohol, then upgrade and imbed in celloidin.

In order to preserve the brownish-black colour of the osmic stain of fat, which soon disappears when the sections are brought to balsam, one may convert the reduced osmic into its sulphide by adding a small quantity of Na_2S to the 70 per cent. alcohol which replaces the fixative. Imbed in celloidin or wax : stain as by the iodine, or better in Best's carmine method, from celloidin. Dr. J. A. Murray informs us that it is generally necessary to stain sections first in warm iron alum, then

warm hæmatoxylin, and then to differentiate in the cold with acid alcohol. Afterwards proceed to Best's carmine.

PAUL BUCHNER (*Praktikum der Zellenlehre I., Berlin*, 1915) fixes overnight in a freshly-made mixture of equal parts of absolute alcohol and strong Flemming. Wash out for two days in 50 per cent. alcohol, imbed in celloidin, stain in Best's carmine.

672. Lison's Method. LISON (*ibid.*) states that contrary to general opinion alcohol is not the best glycogen fixative. He recommends especially for young embryonic tissues the following fluid: dioxan saturated with picric acid 8·5 c.c., formol 1 c.c., glacial acetic 0·5 c.c. He states that Bouin, or better, Bouin-Allen is excellent. It is curious that *in vitro* picric acid does not precipitate glycogen, and Lison believes that the action of the picric acid is to fix the glycogen on some substrate. After Lison's fluid one could proceed straight into pure dioxan, then dioxan wax.

673. Bauer's Method. This method is probably the least specific of all (see BAUER, *Zeit. mikr. anat. Forsch.*, xxxiii, 1933). It depends on the fact that Schiff's reagent gives a violet colour with glycogen which has first been treated with chromic acid. The method has been especially studied by Pasteels and Léonard, as reported by Lison. Fix six hours in Bouin-Allen (adult tissue) or one hour (embryonic and more delicate adult tissues), imbed through dioxan, section. After removing the wax in dioxan, sections are treated in 4 per cent. chromic acid for one hour, or overnight in 1 per cent. chromic. After chroming, the glycogen is insoluble in water, and you wash under the tap for five minutes, then ten to fifteen minutes in Schiff's reagent. Transfer to three changes of SO_2 water as for the "nuclealfarbung" method (§ 623). Wash for ten minutes in running water, counter-stain the nuclei as you wish, mount in balsam. Glycogen reddish-violet, but so may be various polysaccharides, cellulose, starch, tunicin, etc. This is an interesting method.

SHUN ICHI ONO (*Anat. Anthrop. Ass. of China*, 1920) finds that osmicated mitochondrial fixatives preserve glycogen, which can be stained in Best's carmine and iron hæmatoxylin, the mitochondria (grey-black) and the glycogen (reddish) showing side by side.

See also CREIGHTON, *The Formative Property of Glycogen*, London, 1896; GAGE, *Trans. Amer. Micr. Soc.*, xxviii, 1908, p. 203; KATO, *Arch. Ges. Phys.*, cxxvii, 1909, p. 125; BUSCH, *Arch. Intern. Phys.*, iii, 1905, p. 51; MAYER, *Zeit. wiss. Mikr.*, xxvi, 1909, p. 513; ARNOLD, *Sitzb. Heidelberg. Acad. Wiss.*, 1909, p. 1, 1910, p. 3, and 1911, 14 Abh.; *Arch. path. Anat.*, cxciii, 1908, p. 175; *Arch. mik. Anat.*, lxxiii, 1909, p. 265; lxxvii, 1911, p. 346; *Beitr. path. Anat.*, li, 1911, p. 439; FRAENKEL, *Virchow's Arch.*, 1911, p. 197; NEUBERT, *Beitr. path. Anat.*, xlv, 1909, p. 38; ERHARD, *Arch. Zellforsch.*, viii, 1912, pp. 447 and 507; EHRLICH and LAZARUS, *Die Anaemie*, 1898, p. 30; PEKELHARING, *Petrus Camper*, Deel I, 1901, p. 231; DRIESSEN, *Zeit. wiss. Mik.*, xxii, 1905, p. 422; FISCHER, *Anat. Anz.*, xxvi, 1905, p. 399; FIESSINGER, *C. R. Soc. Biol.*, lxvi, 1909, p. 183; NEUKIRCH, *Arch. path. Anat.*, cc, 1910, p. 82.

674. Iron. *Organic compounds of iron*, which are not ionisable into ferric and ferrous ions, and in which the iron cannot be detected by the ordinary reagents, are much more frequently present in animal and vegetable tissues than was previously believed to be the case. In addition to the albuminate compounds, there exist iron compounds giving ferric and ferrous ions,

detectable with the ordinary reagents, and which, for convenience, may be designated *Inorganic Iron Compounds*.

The nature of many of the compounds of iron found in placentas, blood-organs, the liver, etc., is obscure; many of them appear to be formed as degeneration or excretion products, from the breaking down of hæmoglobin. See below.

Most of our knowledge of the methods for the detection of iron in tissues and cells is due to A. B. MACALLUM (*Quart. Journ. Micr. Sci.*, xxxviii, 1895; *Journ. Physiology*, 1897; *Ergebn. d. Physiol. Wiesbaden*, 1908). Macallum has shown that, to detect organic iron, one must convert it into inorganic. This can be done by allowing sulphuric or nitric acid alcohol to act upon sections, or a piece of tissue, for from one to twenty-four hours at 35° C., according to the strength of acid and the size of the object. When masked iron is liberated in the tissues by acid alcohol, most of it is in the form of ferric salts, particularly when the *oxidising* nitric acid is used, and a small part occasionally of ferrous compounds. Inorganic iron compounds in tissues are usually ferric, more rarely ferrous salts.

The commonest tests for iron in tissues are the Prussian blue reaction, Turnbull's blue, and Macallum's hæmatoxylin. *The latter test should never be used alone, because its complete specificity is somewhat doubtful.*

It is hardly necessary to point out that proper precautions should be taken to avoid contamination of the tissue by vessels or chemicals which may contain iron compounds. Glass needles should be used instead of steel, and the water used should have been distilled from a clean glass retort.

The tissues should either be fixed in redistilled formalin (10 per cent.), chemically pure ethyl alcohol or pure 90 to 95 per cent. alcohol, or in redistilled methylated spirit. Bouin's fluid, Flemming, and such mixtures should not be used, as such a practice is almost certain to introduce error. Material should be fixed or hardened for several days in strong alcohol. Sections are made either freehand with a bright rust-free razor *wetted with absolute alcohol*, or by the paraffin method with a dry rust-free knife.

MACALLUM'S HÆMATOXYLIN METHOD. As an indicator Macallum used a 0·5 per cent. solution of absolutely "pure hæmatoxylin" made up in perfectly pure aq. dest. The solution should look *brownish-yellow*, but when alkalies or alkaline earths are added, the colour becomes *violet or red*. When such a pure hæmatoxylin is brought into contact with a *salt of iron*, the yellow colour becomes *blue-black*, or bluish-black; with *organic iron compounds* the *hæmatoxylin is unaffected*. Such compounds must be *unmasked* by sulphuric or nitric acid alcohol as above mentioned.

When the compounds of iron to be investigated are found in tissues, the latter are well hardened in alcohol (purified, *vide supra*), sections prepared and washed in aq. dest., or the tissue simply teased out, and then the hæmatoxylin solution is added. Those parts which go blue-black or blue-violet contain inorganic iron; the remainder of the pre-

paration may go quite dark yellowish-brown, especially nuclei, and the presence of iron may thus be obscured To remove this excess the preparation is treated in a mixture of equal parts of absolute alcohol and ether, but not for longer than one hour. The unaffected hæmatoxylin is extracted, the blue-black compound remains. Clear in oil of cloves, mount in balsam. Such preparations are permanent.

This reaction of inorganic compounds of iron with hæmatoxylin seems to be one of oxidation (Mayer, *Mitth. Zool. Stat. Neapel*, x, p. 170).

Extraordinarily small traces of inorganic iron are thus demonstrated. The method is more sensitive than that of Prussian blue or ammonium sulphide.

Organic Iron Compounds. These will not give the iron reactions unless the complex iron compound has been broken up, that is, the iron "*unmasked*" by some reagent: acid alcohol is used for this. Sulphuric acid alcohol (4 per cent. in 95 per cent. alcohol) and nitric acid alcohol (3 per cent. in 95 per cent. alcohol) are better than the hydrochloric acid alcohol (Bunge's fluid). Sulphuric acid alcohol acts very slowly, especially on bulk tissues, and even Protozoa take twenty-four hours at 35° C. before their masked iron is revealed. Nitric acid alcohol acts more quickly and extracts very little of the iron it liberates (which is a danger with Bunge's fluid); the process is completed in about thirty-six hours.

Sections are treated with acid alcohol, 90 per cent. alcohol, and aq. dest., and then Macallum's hæmatoxylin is added; the sections are washed in aq. dest., stained in safranin, as described in next section, dehydrated and mounted in balsam.

Prussian Blue Reactions on Organic Compounds. Sections after being treated in the acid alcohol (nitric or sulphuric) are washed in pure 90 per cent. alcohol and then in aq. dest. They are placed not longer than five minutes in the following solution: aq. potassium ferricyanide 1·5 per cent., and hydrochloric acid 0·5 per cent. in aq. solution equal parts, freshly made. Again washed carefully in aq. dest. stained in eosin or safranin, dehydrated in alcohol, cleared in oil of cedar and mounted in benzol balsam. The safranin or eosin is used in 1 per cent. strength in 30 per cent. alcohol, for three minutes for eosin, and for one half-hour for safranin, and differentiated in 90 per cent. alcohol.

Ferric and Ferrous Salts both occur in inorganic iron compounds. Ferrous salts may be distinguished from ferric by the fact that only the latter give an immediate reaction with ferrocyanide of potassium, while the former react with ferricyanide of potassium. Fix material in alcohol of about 90 per cent. for several days.

Reaction for Ferric Salts. Wash sections in aq. dest., transfer to 2 per cent. or stronger aq. sol. ferrocyanide of potassium for from three to fifteen minutes. Bring to acid alcohol (1 c.c. in 70 per

cent. alcohol) for about ten minutes. The Prussian blue* reaction takes place. Wash in pure 70 per cent. alcohol, dehydrate, clear, and mount in benzol balsam.

Counter-stain if desired in eosin or safranin (*op. cit.*).

Ferrous Salts. As above, substituting ferricyanide of potassium instead of ferrocyanide, so-called Turnbull's blue.

Simultaneous detection of both categories of salts may be made by using a solution of equal parts of ferricyanide and ferrocyanide.

See also Tirmann, *Goerbersdorfer Veroe Sentl.*, ii, 1898, p. 111; Schneider, *Mitth. Zool. Stat. Neapel*, xii, 1895, p. 208; Carnoy and Lebrun, *La Cellule*, xii, 1897, p. 275; Sumita, *Arch. path. Anat.*, cc, 1910, p. 230; Zaleski, *Zeit. Phys. Chemie*, xiv, 1890; Wassermann, *Anat. Hefte*, xlii, 1910, p. 283; Jones, *Biochem. Jour.*, 1920.

675. Copper. R. Boyce and W. A. Herdman, in their paper on the Green Leucocytosis in Oysters (*Proc. Roy. Soc.*, lxii, 1897—98), have given directions for the application of the well-known potassium ferrocyanide test of chemists, to sections of tissues in which copper is to be detected. These authors fix with proper precautions (*vide supra*, under "Iron") in absolute alcohol, imbed in pure paraffin and cut sections. Care must be taken to avoid acid solutions, such as commercial turpentine or old xylol. Sections are brought from absolute alcohol to distilled water, placed in a 1·5 per cent. solution of freshly-prepared potassium ferrocyanide or, preferably, in equal parts of the same ferrocyanide solution, and a 0·5 per cent. HCl solution, and parts where copper is present go a reddish colour. Sections are then washed in aq. dest., dehydrated in absolute alcohol, cleared in cedar wood oil and mounted in Canada balsam.

Bromine Test for Copper. Mendel and Bradley (*Amer. Jour. Physiol.* xiv, 1905) use a concentrated solution of hydrobromic acid containing a trace of free bromine. Fix as in previous method. Dissolve out paraffin from sections in good xylol, down grade and pass to distilled water. Drain the slide to remove excess water, cover, and introduce a few drops of the hydrobromic acid at the edge. The presence of copper is shown by an intense violet colour. This at once begins to fade and to diffuse throughout the tissue, while the destructive effect of the acid soon obliterates any definite localisation of the metal.

676. Lead. Frankenberger (*Ass. Anat.*, xvi, 1921), Cretin (*ibid.*, xxiv, 1929) fix in neutral bichromate (Regaud fluid does

* **Prussian Blue** [ferric ferrocyanide, $Fe_4(Fe(CN)_6)_3$] made from potassium ferrocyanide and ferric salts.

Turnbull's Blue [ferrous ferricyanide $(Fe_3(Fe(CN)_6)_2]$ made from potassium ferricyanide ($K_3Fe(CN)_6$) and ferrous salts. When prepared under certain conditions it is practically identical with Prussian blue (*Chemical Ency.*, Kingzett, 1928).

well, § 699). Lead is precipitated as yellow lead chromate, easily identifiable in sections.

See also MACALLUM, *Journ. Phys. Cambridge*, xxii, 1897, p. 92; MARFORI, *Arch. Ital. Biol.*, xxx, 1898, p. 186.

For *Zinc* see MENDEL and BRADLEY, *Amer. Journ. Phys.*, xiv, 1905, p. 320.

For *Lime Salts* see GRANDIS and MAININI, *Arch. Ital. Biol.*, xxxiv, 1900, p. 75; SCHAFFER, *Zeit. wiss. Zool.*, lxxxix, 1908, p. 13; LEUTERT, *Enzyk. mikr. Technik*, ii, p. 588; STOELTZNER, *Arch. path. Anat.*, clxxx. 1905, p. 363; MACALLUM, *Ergeb. Phys. Wiesbaden*, vii, 1908, p. 612.

For *Potassium* see MACALLUM, *Journ. Phys. Cambridge*, xxxii, 1905, p. 95; *Ergeb. Phys. Wiesbaden*, vii, 1908, p. 600.

For *Guanin* see GIACOMO, *Zeit. wiss. Mik.*, xxvii, 1910, p. 257.

Concerning the microchemistry of the cell in general, see further *fourth edition;* also CARNOY and LEBRUN, *La Cellule*, xxii, 2, 1897, p. 194; ZIMMERMANN, *Die Morphologie u. Physiologie des Pflanzlichen Zellkernes*, Jena, 1896 (treats also of the animal cell); HAECKER, *Praxis u. Theorie der Zellen und Befruchtungslehre*, Jena; PRENANT, *Journ. Anat. Phys.*, xlvi, 1910, p. 343. For further references see LISON (*op. cit.*).

677. Calcium. The following are two tests for this important substance. Fix in acid-free alcohol or acid-free formol, bring sections to 40 per cent. alcohol, and add 3 per cent. sulphuric acid under the coverslip. Gypsum crystals will be formed if calcium be present. The reaction takes place better in 40 per cent. alcohol, as gypsum is somewhat soluble in water (SCHUEJENINOFF, *Zeit. wiss. Micr.*, 1897).

Crétin's Colour Test. This is an extremely sensitive test for giving a specific blue colouration. Other metals give colours with this test, for example, barium and strontium, green; iron, brownish-violet; magnesium, rose; silicon, yellow.

Mix in a mortar 1 part of trioxymethylen and 2 parts of gallic acid. For the test dissolve 0·25 grm. of the mixture in 5 c.c. of boiling distilled water. To this boiling solution carefully add 0·5 c.c. of ammonia 18° B., shake till a straw-yellow colour appears. It is then ready, but if it goes brown or rose colour it is useless, and in any case only lasts a short time. Now remove the wax from sections with xylol, and wash off the xylol with chloroform; throw off excess chloroform, add the gallic acid reagent and after ten or fifteen seconds, tip off excess reagent and leave exposed to the air. The characteristic blue colour should appear if calcium be present.

This method is not easy, and like other complicated and ill-understood microchemical reactions, requires practice (see *Bull. histol. appl.* No. 3, 1924). Masked calcium is detected best by microincineration (see §§ 678, 1269)

677a. Vitamin C. The researches which led to the elaboration of a cytological technique for this vitamin were initiated by A. Szent-Gyorgyi in 1927. Adrenal gland cortex treated with $AgNO_3$ solution rapidly blackens. A crystalline substance, "hexuronic acid," was isolated, and finally identified with vitamin C, partly with the assistance of two Americans, C. KING and W. A.

WAUGH (*Bio. Chem.*, vol. 97, 1932). In 1932, MOORE and RAY showed that in scurvy the silver nitrate reducing property of the adrenal disappeared. Finally, in July 1933, the Australian investigator, G. BOURNE, published the first cytological account of vitamin C. The technique was improved by GIROUD and LEBLOND (*Arch. Anat. Micr.* 1934, *C.R. Ass. Anat. Bruxelles*, 1934). They showed that this vitamin was localised in cells in connection with the mitochondria and Golgi apparatus, and the questions thus raised are of prime importance to cytologists.

G. Bourne has carefully investigated the cytological technique for this vitamin and recommends the following procedure: Fix tissues in a solution of 5 per cent. $AgNO_3$, to 100 c.c. of which 5 c.c. of glacial acetic acid have been added (modified solution of Giroud and Leblond). For Protozoa, etc. smears, a few drops of the solution are added to the liquid on the slide with the cells. Examine after ten to fifteen minutes. Or better, isolate organisms in centrifuge, treat *en masse* with solution, wash in pure glass distilled aq. dest., treat in photographic " hypo." Wash several times in aq. dest. upgrade in ethyl alcohol, clear, imbed and section if necessary. *The best results are obtained by carrying out impregnation in darkness.* This is important.

For animal tissues Giroud and Leblond washed out the blood from the dead animal with an isotonic solution of levulose, and injected with acetic acid silver nitrate solution (10 per cent. $AgNO_3$, with the addition of 0·5 c.c. of acetic acid for each 100 c.c. of solution). The silver nitrate was run out with distilled water, followed by injection of " hypo." The hypo. was washed out, pieces of tissue removed and sectioned by the usual wax method.

A. BOURNE'S **Method for Simultaneous Demonstration of Vitamin C and Fats.** Cut fresh tissue by freezing technique. Drop into slightly acid 5 per cent. $AgNO_3$ for a few minutes in the dark. Wash in water for a few minutes, pass to Sudan in 90 per cent. alcohol. Mount in glycerin jelly.

Finally, it should be mentioned that vitamin C may exist in (*A*) reduced form, reacting readily with $AgNO_3$, and (*B*) reversibly oxidised form not so reacting. The ordinary technique simply demonstrates form (*A*).

To get over this difficulty Bourne exposes pieces of tissue to vapour of glacial acetic acid for some minutes, cuts thin slices and leaves in atmosphere of hydrogen bisulphide for fifteen minutes. This converts form (*B*) into form (*A*). All trace of hydrogen bisulphide *must be removed.* This is done *in vacuo* until most of the H_2S has been sucked out of the tissue (fifteen to thirty minutes). The sections are then exposed to a strong stream of nitrogen gas for fifteen minutes. Before concluding that vitamin C is not present the hydrogen bisulphide method should be used. Actually

there may be little difference between the various methods given when one examines the slides.

Validity of Method. This is recognised widely as a valid method. The vitamin becomes black with silver nitrate and is found in the form of scattered granules. For its connection with the Golgi apparatus and mitochondria see BOURNE (*Aust. Jour. Exper. Biol.*, 1936). The investigator will need to be careful in using this silver nitrate method. The reagents should be pure, and the vessels cleaned. At the present time no substance other than vitamin C is known which in the dark is capable of reducing the silver nitrate acetic acid solution.

678. **Microincineration.*** This consists of incinerating paraffin sections or smears in an electric furnace so that the organic material is removed and the inorganic ash remains. The method is elegant, but *strictly limited in application.* It was introduced into histology by the distinguished French histologist, A. Policard. The reader can obtain an idea of the remarkable precision of the microincineration method by consulting the paper by Horning in the Report of the Imperial Cancer Fund Laboratory, 1935, where a suitable electric furnace is described, and where a number of excellent microphotographs is given.

Microincineration is applicable to research on insoluble mineral salts especially. It does not generally enable one to distinguish between various mineral elements, as nearly all form a white ash. Iron gives a red ash when it becomes the oxide (Fe_2O_3). A reasoned evaluation of the method will be found in Horning's paper (*op. cit.*).

See also POLICARD, *Bull. Hist.*, i, 1924 ; *ibid.*, iii, 1926 ; *ibid.*, iv, 1927 ; POLICARD and OKKELS, *Anat. Rec.*, xliv, 1930.

* See § 1269.

CHAPTER XXX

CYTOLOGICAL METHODS: GOLGI BODIES * MITOCHONDRIA † YOLK ETC.‡

679. In recent years some doubt has arisen as to what is the Golgi apparatus. Before proceeding to a description of the various methods for demonstrating this and other bodies in the cell cytoplasm it will be necessary to enter into some explanations.

What is the Golgi Apparatus? The Golgi apparatus is constituted typically in the higher vertebrate neurone by a network, more or less complete, of argentophile and osmiophile substance lying in the cytoplasm. In young neurones the network is situated on one side of the nucleus in the so-called juxta-nuclear excentric position. In tissues which have been fixed in formalin, immersed in silver nitrate solution, and then reduced in hydroquinone solution, the substance of the Golgi apparatus often, but not always, becomes black with reduced silver. This phenomenon can be made to occur more certainly by adding arsenious acid, cobalt or uranium nitrate, or cadmium chloride to the formalin fixing solution. Similarly, pieces of tissue fixed in osmium tetroxide solution, and left in it for some weeks at room temperature, gradually show a blackened area in the same place in given cells as with the formalin silver techniques. In some, but not all cells, certain vital dyes segregate in vacuoles or granules in the region of the Golgi apparatus. In other words, the area in which the Golgi material lies is in such cases a functionally specialised part of the cell. Summarised, tests for the Golgi apparatus are as follows:—(*a*) It goes black in the formalin silver nitrate and osmic Golgi apparatus methods. (*b*) *It does not stain in neutral red.*** (*c*) It passes centripetally and is found below the fat, in ultra-centrifuged cells (§ 732).

In metazoan cells (including sponges, Parazoa), the Golgi apparatus is definitely identifiable. With the exception of the Sporozoa, in the Protozoa there is still no consensus of opinion as to whether true Golgi material is present. The Golgi apparatus of Protozoa does not appear to us to be the neutral red staining granules, though in Metazoa neutral red staining substance

* Chondriosomes, chondriokonts, plastochondria, "chromidia," bioblasts, chondriome, chondriomites, etc., etc.

† Nebenkern batonettes, idiozome rods, "Golgi-Kopsch apparat," apparato interno reticolare, dictyosomes, Binnennetz, etc.

** See footnote § 765; the invertebrate dictyosome apparently does sometimes stain. One, at least, authentic case is the Scorpion dictyosome.

‡ By J. B. G.

may be closely associated with Golgi material. The writer holds the view that in some flagellated organisms, the Golgi apparatus is associated with the base of the flagellum, as in sponges. For most recent literature see PATTEN and BEAMS (*Q.J.M.S.*, 1936), HALL (*Bot. Review*, Feb. 1936).

680. What is the "Vacuome"? This is the French word for vacuole, and like the German term "nebenkern" has no universally recognised meaning in Cytology. It has been used especially by P. and P. A. Dangeard, and A. Guilliermond for certain plant vacuoles, which in some cases stain in neutral red. In 1924–1927, the late Dr. M. Parat endeavoured to homologise his artificially produced nets and vacuoles in Chironomus salivary glands, with the Golgi net of neurones, and the neutral red stainable "vacuome" in certain plants. In animals many cells segregate neutral red both supra-vitally and intra-vitally. In some cases the neutral red dissolves or is segregated within pre-existing granules or vacuoles (Ehrlich), in others, the globules and nets are neo-formations (Parat), in still others both types appear in one cell and, finally, by some interaction between the dye-stuff and the protoplasm, insoluble bodies called "Krinom" (Chlopin) become formed. Then with regard to those pre-existent granules which do stain vitally in neutral red, there is little or no evidence that they are homologous structures in various plant and animal cells. Volutin granules, fuchsinophile granules, chromatic and metachromatic granules, pre-zymogen granules, single granules in insect and other spermatids are only a few of the assemblage of formed bodies which become red in this dye. Some of these bodies certainly go black in the osmic * and silver methods, as well, and it appears to us just as unjustifiable to call them Golgi apparatus, as to classify all granules which stain vitally in neutral red as "vacuome."

Owing to this confusion, and to the fact that we are quite unwilling to promote the "vacuome" to the status of a definite and constant cell inclusion like the Golgi bodies and mitochondria, we do not propose to use this word. In a separate chapter (§ 766), the methods for staining or producing neutral red vacuoles are described. For a summary of Dr. M. Parat's techniques see § 733.

681. The Mitochondria, Chondriome. The mitochondria are numerous granular, filamentous or rod-shaped cell inclusions which stain blue-black in iron hæmatoxylin after formalin, chrome, chrome-osmium fixation, and are usually dissolved or much distorted by alcohol and acetic acid containing fixatives. In some organs, *e.g.* liver, or mitochondrial middle-piece of insects, the remains of the mitochondria show clearly even after Carnoy fixation.

Mitochondria are, in animal cells, the heaviest of the cell

* Stain in Neutral Red, then apply osmic acid solution. The vacuoles often blacken in a few hours, but this cannot be considered a test for the Golgi apparatus.

inclusions, do not stain in neutral red (except in the lethal stage), and especially in vertebrate cells stain specifically in Janus Green. In the male metazoan germ cells, the mitochondria form the middle-piece, the Golgi bodies secrete the acrosome. The only exception to this is in the sperms of Peripatus where the mitochondria only doubtfully form a small middle-piece. Here, however, the acrosome is formed normally from a Golgi body. In seeking a discrimination between Golgi bodies and mitochondria it is useful to make slides of the germ cells, and necessary to use the ultra-centrifuge where doubt may arise.

682. The Validity of Preparations showing Golgi Bodies and Mitochondria. Preparations which show Golgi bodies and mitochondria are regarded as valid for the following reasons :—(*a*) The stained preparations correspond in every way with living cells in which these cytoplasmic inclusions may be seen, *e.g.* testicular cells of insects such as *Gryllus* and *Lepisma*, or of molluscs such as Helix aspersa. (*b*) The cell inclusions undergo definite and fixed movements in secretion (*e.g.* pancreas, thyroid), in spermatogenesis, and in mitosis of the cell. (*c*) The cell inclusions may be centrifuged into layers by the ultra-centrifuge of Beams.

683. Study of Golgi Bodies and Mitochondria, Vitally. This may best be carried out in molluscs such as Helix aspersa or insects such as *Gryllus* or *Lepisma,* in which both types of inclusions are to be seen *supra vitam* in the spermatogenic cells especially. The requisite organ should be dissected out in appropriate salt solution (§ 1430) and after teasing, if necessary, mounted in a hanging drop preparation. (See GATENBY, *Proc. Royal Society of London*, Vol. 104B, 1928; R. N. MUKERJI, *Jour. Royal Microscopical Society*, Vol. 49, 1929; and H. HERBERT JOHNSON, *Zeit. für wiss. Zool.*, Vol. 140, 1931.)

The appearance of living cells may be compared with the images got with Weigl, Kolatchew and Flemming-without-acetic acid, or Champy iron-alum hæmatoxylin slides. In vertebrate cells it is less easy to identify these inclusions in the living cell, but the regions which impregnate or stain with the methods described in this chapter may be ultra-centrifuged into distinct layers. For the ultra-centrifuged Golgi bodies, etc., of Sporozoa, see Miss M. DANIELS, *Quart. Jour. Micr. Sci.*, 1937.

684. Methods for Cytoplasmic Inclusions (General). The mitochondria and Golgi apparatus never clearly appear in stained sections prepared by such methods as fixation in corrosive acetic, Gilson, Bouin, Carnoy or Flemming-with-acetic acid, and staining in Ehrlich's hæmatoxylin and eosin, toluidin-blue and eosin, paracarmine and borax carmine. Though the mitochondria and Golgi apparatus are properly fixed by formalin, Müller, Flemming-without-acetic acid, Champy, Altmann, etc., they will rarely appear visible in stained sections which have been

prepared in Ehrlich's or Delafield's hæmatoxylin or carmine stains, or in fact with any of the current laboratory stains used for general zoological purposes. The mitochondria and Golgi apparatus may appear visible in sections fixed in formalin, Müller, etc., and stained in Altmann's acid, fuchsin-picric acid, iron-hæmatoxylin, Benda's alizarin and crystal-violet, etc. The Golgi apparatus rarely becomes visible after any of the above methods, and to study it one must use more specialised methods; to study the Golgi apparatus and the mitochondria by routine zoological laboratory technique is not possible, simply because these methods will not demonstrate the bodies in question. Nearly all of the older fixing mixtures contain either alcohol, chloroform, or acetic acid, but the last few years of cytological research have shown that the picture given by a fixing mixture containing them is incorrect and inadequate, and one cannot fail to be surprised at the improvement produced when these reagents are omitted. Nearly all modern research on the cytoplasm has to be carried out by using chrome formalin or osmium fixatives, followed by iron-alum hæmatoxylin, Benda's crystal-violet, or Altmann's acid fuchsin; or by the important Kopsch and Mann-Kopsch, and Ludford osmium tetroxide methods; or by the useful methods of Cajal, Golgi or Da Fano's modification of Cajal, which consist of silver nitrate impregnation following formalin fixation. *Intra-vitam* methods, such as janus green, neutral red, or dahlia violet, are also used extensively. The mitochondria are extremely fuchsinophile, and after chrome-osmium fixation stain strongly in iron-alum hæmatoxylin. The Golgi apparatus of somatic cells and of ovarian cells rarely stains by these methods (Altmann or Heidenhain) unmodified, although the Golgi apparatus of the male germ cells nearly always stains in fuchsin or hæmatoxylin after chrome-osmium or formalin fixation.

In § 648 is a special article on fats and lipoids, and on methods for their study; on the following pages are set forth various techniques for the investigation of *definite cell organs* known to be partly lipoid in nature. The application of all these methods to embryological study opens the way to a valuable field for research. Fats or lipoids form a special part of almost all cell-organs, as seems to be indicated by fixing tests, and so far as we know such substances are always intimately associated with protoplasm. Many of the lipoids appear to be able to form with certain metallic salts or oxides such as CrO_3, $K_2Cr_2O_7$, $PtCl_4$, OsO_4, etc., compounds insoluble or only slowly soluble in alcohol or such clearing oils as xylol, benzol, or chloroform; this is one of the several reactions which take place when a cell is fixed in such a fluid as that of Flemming (without acetic acid), Champy, or Altmann, and subsequently dehydrated and cleared.

685. Choice of Method.* We have given below a number of methods for lipoid granules, mitochondria, and other cell inclusions, and not all are suitable for every piece of work. It is very rare to find that one single method will produce the same good result in both vertebrate and invertebrate tissues. In the same way, methods which act satisfactorily with amphibia will often give disappointing results with mammalia. Osmic-chrome fixation will nearly always be found excellent for all classes of invertebrata; Flemming-without-acetic acid and Champy-Kull can be highly recommended. For amphibia the addition of some $K_2Cr_2O_7$ to the Flemming is necessary before a correct fixation of the mitochondria is obtained; thus Champy's fluid was invented for amphibia and gives very satisfactory results (§ 49). For mammalian tissues a *preliminary* fixation in osmic acid fixatives is not generally indicated; the tissues of mammals are far more "fatty" than those of invertebrata or amphibia, and one finds that the OsO_4 becomes reduced very rapidly and penetration is very poor. For mammalian tissues formalin-chrome (Regaud, Bensley-Cowdry), formalin-corrosive or formalin alone are indicated as a preliminary treatment. Formalin does not destroy lipoids, and by subsequently placing small pieces of chrome-formalin fixed tissues in osmic acid (post-osmication), a fixation of lipoids and fats is obtained (Schridde); the same result may be got by fixing tissues in chrome salts and then transferring the osmic acid. It should be noted, however, that previous fixation in a chrome salt often prevents the blackening of the Golgi apparatus unless the chrome salt is well washed out of the tissues, and the osmication is prolonged; the methods of Nassonow and Ludford should be used when an impregnation of the Golgi apparatus is required by means of an osmic method, but the formalin silver nitrate methods of Aoyama and Da Fano are always clearly indicated for work on the Golgi apparatus of mammalian tissues. Fixation of tissues in the fluid surrounded by crushed ice should be tried (§ 626). So far as possible *intra vitam* and fresh smear preparations should be used, as these nearly always give valuable results.

686. Specificity of Techniques for Cytoplasmic Inclusions, Fats, and Lipoids. As a rule the lipoid granules, vacuoles, and cell organs containing fats or lipoids are formed, not of one pure substance, but of a mixture of several. Consequently it is necessary to proceed with caution in claiming a specificity for the techniques for various lipoid substances: properly used, however, these methods may give valuable evidence as to the precise nature of any special body: micro-chemical methods, which

* The beginner is recommended to master such techniques as those of corrosive acetic and borax carmine, or Zenker and Ehrlich's hæmatoxylin, before trying these methods.

depend for their application on the use of complicated fixing and staining methods, are to be used cautiously. For example, Benda (§ 697) and Altmann-Bensley methods (§§ 694 and 701) will stain granules other than mitochrondia, while the Cajal formalin uranium and silver nitrate technique impregnates bodies apart from the Golgi apparatus. In all these cases, however, the number of exceptions is small, and suitable differentiation between two doubtful bodies can be made by some other method.

Over-impregnations or impregnations done with disintegrating or unsuitable osmic or silver nitrate fluids produce preparations in which not only the mitochondria but even cell walls and nuclei are more or less blackened. It would be incorrect to state that only experienced technicians are able to work these methods so as to get the degree of specificity expected by those who use these methods for research work. In molluscan gonads, the tissues of young mammals, insect gonads, etc., perfectly good preparations may be made by beginners, and the degree of specificity (which is quite considerable) judged.

687. On Killing Animals for Cytological Purposes. So far as possible avoid narcotics of any sort. Either cut off the heads of invertebrates, or, if delicate like some worms, drop them whole in the fixative; kill vertebrates by a blow on the head, or by pithing. If for a study of brain, bleed, or anæsthetise in coal gas, less preferably chloroform or ether. Insects can be killed with cyanide or xylol.

Hints on removing Tissues and Cutting. Avoid pinching the material with forceps, as this is said to introduce artifacts; it is preferable to remove tissue without recourse to dissection under tap-water or salt solution; for Kopsch techniques, quickly remove blood or lymph, etc., from surface of material with aq. dest. before placing in fixer; for cutting tissue the best instrument is a new safety razor blade stuck in a special holder made for the purpose, or in a split penholder, or held by artery forceps. When working on arthropods, it is best to dissect the organ from the animal, instead of preserving the whole body; surrounding fat, etc., should be removed. See also §§ 10 and 628.

688. Washing Out for Cytological and Histological Purposes. The beginner is often puzzled by the problem of how long to wash out after fixatives. Elsewhere (§ 35) we have mentioned that usually picric and corrosive material is washed out in alcohol, whereas chrome fixed tissue is treated with tap-water. This is not all, however, and *the washing out will often be found to have important results on the subsequent staining; e.g.* small animals like daphnids, fixed in Champy's fluid overnight, must only be washed out a few minutes, or under an hour. If such washing is prolonged too much, the subsequent hæmatoxylin stain will be a failure, because it seems necessary to leave some chrome-osmium in the tissues to make staining a success (§ 706). Larger objects,

or objects left for days or weeks in chrome or osmium, can be washed out overnight or longer. This hint with regard to failure to stain properly in hæmatoxylin or fuchsin may enable the beginner to trace the fault to its source. Often, however, the stain itself is bad.

Washing is best carried out under the tap by covering the vessel with gauze, held on by a rubber band. Minute objects wash out more quickly and can be done by changing distilled water by means of a pipette until the objects are properly washed.

689. Difficulties and Faults in Techniques for the Cytoplasmic Inclusions. It is important for beginners not to leave out the Aoyama or Da Fano methods in favour of the Weigl or Kolatchew: the reason for this is that failure of the latter methods (so far as the Golgi apparatus is concerned) is sometimes complete while the preparations may appear quite good. Failure may be brought about by various factors; some we understand partly, some not at all. For example, occasionally specimens of commercial osmium tetroxide will not impregnate the Golgi apparatus; in some cases this is caused by not washing out the chrome fixative sufficiently; but this is not the only cause, as in Weigl's corrosive sublimate method failure is also sometimes complete. Therefore, as a control on these osmic methods, Aoyama or Da Fano methods should be used.

It should be unnecessary to point out that the pieces, or animals used, should not be too big, and the dishes, capsules or phials should be properly cleaned. When washing out under the tap it is necessary to make sure that the water will run overnight. One cannot counterstain osmicated preparations of the Weigl or Kolatchew type unless one has carefully treated the sections in permanganate of potash and oxalic acid, or in hydrogen peroxide. One may, of course, stain the nuclei in acid neutral red (§ 710), but one cannot stain the mitochondria properly unless bleaching has been done. It is possible to get interesting preparations from pieces of animals which have been killed for dissection, but one cannot depend on such material for research work. For the best results animals which have just been killed without ether or chloroform should be used.

For oogenesis studies and in protozoology we believe that the ultra-centrifuge is indispensable. It is better not to take up the study of granules in eggs or protozoa, unless it is possible to have some material ultra-centrifuged. Sometimes one can make quite good preparations of mitochondria and Golgi bodies from material which has been fixed in 10 per cent. formalin and left in it for months. But if the formalin has disintegrated badly, such material is useless. In the formalin silver methods, fresh formalin, and newly-made solutions of silver nitrate, and new reducer are recommended. The commercial formalin used for museum work,

and sometimes supplied in laboratories, is not good for cytology. For some reason old solutions of silver nitrate will not always work properly. Old reducer is useless. It should be remembered that there is no recognised intra-vital stain for the Golgi apparatus. *The globules which sometimes stain up with neutral red are not the Golgi apparatus.* It is much better not to endeavour to investigate the cytoplasmic inclusions of Protozoa until one has had some experience with Metazoa. It is safer to study some vertebrate and mollusc or insect material first. It should be noted that in many cases both mitochondria and Golgi bodies can be seen in the living cell (*e.g.* mollusc or insect germ cells)—this will be the surest index of their actual shape and size. Living cells are best examined in the natural fluid of the animal, but if this is not possible, refer to § 1430, where various suitable media are mentioned.

690. Advances in the Techniques since 1928. Reference to cytological memoirs dealing with the cytoplasmic inclusions, shows that the methods have largely become stereotyped to Da Fano, Kolatchew, Weigl, Regaud-acid fuchsin, and Champy iron-alum hæmatoxylin. Some workers have depended solely on Weigl or Kolatchew, and Regaud alum hæmatoxylin or acid fuchsin. The last two methods, namely pre-fixation in chrome-osmium or corrosive-osmium followed by post-osmication and fixation in Regaud's chrome-formalin followed by alum hæmatoxylin or acid fuchsin, will certainly cover most of the ground, but there are some recent improvements which could be noted with advantage. Firstly, the formalin and chrome-fixatives should be made isotonic : while this may not improve the fixation of surface cells, it will give a larger amount of material for study. Second, F. Aoyama's* modification of Cajal, is undoubtedly an improvement on Da Fano, which has gradually ousted Cajal's method. Third, our ideas on the subject of the microchemistry of fats have had to undergo considerable change. Cytologists should consult Chapter XXVIII for a modern report on this subject, which is of major importance for them.

691. Chrome-osmium Techniques. Potassium bichromate or chromium trioxide, used in watery solutions, will not alter true fats in such a way that full vacuoles of the latter will appear in finished sections prepared by routine methods; but combinations of such salts with osmium tetroxide provide fixatives which will preserve almost all cell elements in finished sections. *It should be carefully noticed* that the long osmication of the Weigl-Kopsch method will not always prevent fat globules from being dissolved out by xylol alcohol used for clearing and mounting. Such preparations when being used for studies of fats must be mounted in Farrants' or some such watery medium

* Refer to 730a, also.

(see Miss J. C. Hill, *Archiv. f. exper. Zellf.*, 1936, and T. J. Macdougald, *ibid.*). So far as we are aware, this warning only applies to the delicate fat globules in fibroblasts *in vitro*.

The basis of all chrome-osmium techniques consists in a preliminary fixation of small pieces of tissue, small embryos or eggs, in such a fluid as Benda, Champy, Flemming-without-acetic acid, or Altmann, for from at least twelve hours to a week. The osmic reaction is then, in some methods (Kull, Benda), "set" or strengthened by the reducing effect of pyroligneous acid; following this treatment is a further "chroming" in 3 per cent. bichromate of potash, and, finally, a thorough wash out under the tap. Material treated in this way is generally perfectly preserved, and fit for selective staining. Arranged below are chrome-osmium techniques of progressive intensity and difficulty.

692. Modified "Flemmings" for Cell Inclusions. Benda: 15 c.c. chromic acid 1 per cent., 4 c.c. osmic acid 2 per cent., 3 to 6 drops of acetic acid. Meves: 15 c.c. of chromic of 0·5 to 1 per cent., containing 1 per cent. sodium chloride, with 3 to 4 c.c. of 2 per cent. osmic acid, and 3 to 4 drops of acetic (*Enzyk. mik. Techn.*, 1910). Strong Flemming-without-acetic acid; and same solution diluted by one-half or one-third (Gatenby, *Quart. Journ. Micr. Sci.*, 1919). The presence of a small quantity of acetic acid is always liable to introduce distortion, but less so among vertebrates than among invertebrates.

In § 47, a method for making up salt Flemming-without-acetic, and in § 49 salt Champy is given, and should be tried. See also Bensley, § 48, and Baker and Thomas' modification of Altmann's fluid, § 48. Lison (*ibid.*) attributes Flemming-without-acetic to Heitz (?). Meves sometimes used Flemming-without-acetic, but preferred some acetic acid. The method given below is original so far as times are concerned, and these should be rigidly adhered to for the best results.

693. Gatenby's **Flemming's Strong Fluid without Acetic Acid, and Iron Hæmatoxylin.** Small organs freshly dissected out in normal saline, or parts of organs cut with safety razor blade, not more than 5 mm. in diameter, are placed in about 15 c.c. of one of the above-mentioned fixing fluids. A glass-covered capsule or vial is the best vessel to use, and the material is left for at least twenty hours, and not longer than one week. We find about twenty-four hours gives a satisfactory fixation of most tissues. After fixation the liquid is poured away, and the material is washed for at least two hours, and not necessarily longer than five, in running tap-water. It is then passed through up-graded alcohols, beginning at 30 per cent., giving the material at least three hours in the strengths 30, 50 and 70 per cent., and overnight in 90 per cent. The pieces of tissue are dehydrated two or three hours in two changes of absolute alcohol, and then transferred to a mixture of half absolute alcohol and half xylol for a quarter of an hour; then pure xylol, and imbedded in wax. Sections are

cut from 4 to 8 μ, but we generally find 6 μ to be convenient. *Leave eight to ten hours in iron alum, twelve to twenty hours in hæmatoxylin.* The most convenient method is to leave all day in alum, and overnight in hæmatoxylin, differentiating next morning. This method gives a delicate and precise stain of the mitochondria (and Golgi apparatus or nebenkern batonettes of male germ-cells only), fatty yolk is black, while yolk is generally greenish-brown. Especially recommended for germ-cells, and histology of Invertebrata, but with vertebrate tissues, and especially mammalian material (not embryos), it often gives poor results; for such material, Helly, Zenker, or Regaud's methods are indicated (Gatenby, *Quart. Journ. Micr. Sci.*, 1919), or fix in salt Flemming § 47.

Note that Flemming-without-acetic acid is not a suitable fixative for after-staining in Altmann's acid fuchsin. For this the material must be washed in distilled water for a short time after fixation, and then transferred to 3 per cent. bichromate of potash for three days; or the more elaborate mordanting as for Champy-Kull may be used (§ 695).

694. Altmann's **Acid Fuchsin and Picric Acid** (*Die Elementarorganismen*, Leipzig, 1890). Fix twenty-four hours in mixture of equal parts of 5 per cent. bichromate of potash and 2 per cent. osmic acid. Imbed in paraffin, stain sections on slide for one minute over flame, with a solution of 20 grm. (*sic*) of acid fuchsin in 100 c.c. of aniline oil-water *. Cool, and wash out in a saturated alcoholic solution of picric acid diluted with 2 volumes of water, heat being used as before to aid differentiation; blot, dip into 90 per cent. or absolute alcohol, xylol, balsam. This method only stains granules which can be seen *intra vitam*; properly used it never produces artifacts, and Fischer's critique is quite wrong (*Fixirung Faerbung u. Bau des Protoplasmas*). Altmann's original method has been superseded more or less by the following method of Champy-Kull.

Both Murray and Gatenby found that the 20 grm. of acid fuchsin would not dissolve in 100 c.c. of anilin oil-water; only about 5 to 7 grm. would dissolve, and this quantity will make a perfectly efficient solution. John Baker gets 12 per cent. to dissolve, Conn's specimen up to 20 grm.

J. Baker and M. Thomas (Baker, *Cytological Technique*, 1933) propose the following modification: equal parts of 3 per cent. bichromate of potassium and 2 per cent. osmium tetroxide. Fix for four days, or for one day and post-chrome for three days in 3 per cent. bichromate alone. Wash out overnight in running water. Cut sections 5 μ or less. Add the acid fuchsin, heat with Bunsen till it steams for one minute. Leave five minutes to cool. Pour off, then distilled water, then flood with picric solution

* Add oil to water till no more dissolves, shake vigorously, filter.

(20 parts of saturated picric in absolute alcohol and 80 parts of 20 per cent. alcohol). The red stain begins to be extracted at once. After half a minute examine under microscope. The nuclei should be distinctly yellow, mitochondria still bright red. If differentiation is nearly complete, transfer to a weaker picric solution (as above, but only 10 parts of picric to 90 parts of 20 per cent. alcohol), which slows down the action and enables it to be watched more carefully. Wash in aq. dest., then quickly into 70 per cent., 90 per cent. and to absolute alcohol (in which two minutes), xylol, mount .

695. CHAMPY-KULL'S **Acid Fuchsin, Toluidin Blue and Aurantia** (KULL, *Anat. Anz.*, Bd. xlv, 1913). The following method, while being generally useful, will be found very convenient for work on Invertebrata. It gives results intermediate between those of Benda and Altmann, but is shorter and undoubtedly better than the method of Benda. It will be found very useful for embryological research, and probably also for protozoology. Fix in Champy (§ 47) (we find Flemming-without-acetic acid will do, too) for twenty-four hours. Pieces to be fixed must be small. After fixation wash half an hour in aq. dest., and then transfer to a mixture of 1 part acid acet. pyrolignosum rect., and 2 parts 1 per cent. chromic acid, for twenty hours. Wash half an hour in aq. dest., and transfer to a 3 per cent. solution of potassium bichromate for three days. Wash under tap for twenty-four hours; pass through up-graded alcohols to xylol; imbed in paraffin wax (or celloidin method, if desired). Section 4 or 5 μ. Proceed as follows : (1) Stain in Altmann's acid fuchsin anilin oil mixture (5 to 10 grm, of acid fuchsin in 100 c.c. of anilin oil-water), and heat till steaming. (2) Set slide aside to cool for six minutes (this is important), pour off, and wash quickly in aq. dest. (3) Counter-stain in either a 0·5 per cent. solution of toluidin blue or a saturated solution of thionin in aq. dest. for one to two minutes. Wash in aq. dest. In some cases the time in the blue stain must be shortened. Transfer to a 0·5 per cent. solution of aurantia in 70 per cent. alcohol for from twenty to forty seconds, watching extraction of fuchsin stain under microscope. Differentiate the blue stain in 96 per cent. alcohol, then absolute, xylol, and balsam. The chromatin is generally blue, mitochondria (and occasionally Golgi apparatus) are red, and the ground cytoplasm is golden-yellowish to green. This modification of Altmann's method is a most brilliant three-colour stain which is highly recommended. We have found that it is useful for histological as well as cytological purposes; sections of Annelids, or of flat-worms, for instance, prepared by Champy-Kull show beautiful colour gradations in their different tissues. The preparations begin to fade after a year.

After Champy-Kull fixation you can : (*a*) stain in iron hæma-

toxylin (long method, § 693), (*b*) stain as for Benda (§ 697), (*c*) mount unstained for examination of osmicated granulations, (*d*) stain in safranin and light green (§ 344). For a chart illustrating Champy-Kull technique, see below.

MAXIMOW (*C. R. Soc. Biol.*, Paris, lxxix, p. 462) fixes in Champy, washes slightly in water, transfers to mixture of 1 per cent. chromic acid 1 part, glacial acetic 2 parts, for twenty-four hours. Wash again for half an hour, place for three days in 3 per cent. $K_2Cr_2O_7$. Wash in running water. Stain sections as above.

696. Champy-Kull Fixation (Germ-Cells)

Subsequent method.	Mito-chondria.	Fat.	Yolk.	Golgi apparatus.	Nucleus.
Mount unstained	Yellowish.	Black (extractable in turpentine).	Yellow to black.	Yellowish or does not show.	Yellowish.
Champy-Kull stain	Red or pink.	Black.	Yellow. to black.	Generally will not show in somatic cells or ovaries, red in male germ cells.	Chromatin blue to greenish (nucleolus red).
Iron hæmatoxylin	Black.	Black, provided it has not been extracted in turpentine.	Same as above.	Same as above, but black in male germ cells.	Chromatin grey to black (nucleolus black)
Benda stain (alizarin and crystal violet)	Violet.	Same as above.	Same as above.	Same as above, but violet in male germ cells.	Chromatin brown or yellowish (nucleolus violet).

697. BENDA'S **Alizarin Method** (*Ergebnisse der Anat.*, xii, 1902 (1903), p. 752, and other places) is as follows :—Harden for eight days in strong liquid of Flemming, the acetic acid therein being reduced to 3 drops (or as for Champy-Kull or Regaud). Wash for an hour in water and put for twenty-four hours into a mixture of equal parts of pyroligneous acid and 1 per cent. chromic acid, then for twenty-four hours into bichromate of potash of 2 per cent., wash for twenty-four hours and imbed in paraffin. Sections on the slide are mordanted for twenty-four hours with 4 per cent. solution of ferric alum or diluted *liq. ferri sulfur. oxydat.*, then rinsed with water and put for twenty-four hours into an amber-yellow aqueous solution of Kahlbaum's sulphalizarinate of soda, prepared by dropping 1 c.c. of saturated alcoholic solution thereof into 80 to 100 c.c. of water. Rinse in water, flood the slides with the solution of crystal violet (§ 357) diluted with an equal volume of water, and warm till vapour is given off. Rinse, differentiate one or two minutes in 30 per cent. acetic acid (till the nuclei come out reddish), wash in running water for five to ten minutes, dry with blotting-paper, dip into absolute alcohol, pass through bergamot oil into xylol and balsam.

Mitochondria violet, chromatin and "archoplasm" brown-red, certain secretion granules pale violet, centrosomes red violet.

Instead of the staining solution prescribed above (which may be kept in stock) you may take (*Enzyk.*, ii, p. 198) a *freshly prepared* mixture of equal parts of anilin water and saturated alcoholic solution of crystal violet—and this is to be preferred.

Dr. John Baker informs us that the following three suggestions may be found helpful :—

1. Cut very thin sections, the thinner the better.
2. Add 3 drops of 1 per cent. calcium acetate solution (aqueous) to the slide-bottle containing the alizarin solution. This favours the action of the stain.
3. While differentiating with acetic acid, disregard the interior of the piece completely and concentrate the attention on the part that has been well fixed by the osmium.

Some workers prefer to harden as Benda, but to stain with **iron hæmatoxylin** instead of by the alizarin process ; the special hardening rendering the hæmatoxylin stain sufficiently specific.

Arnold (*Arch. Zellf.*, viii, 1912, p. 256) stains first with iron hæmatoxylin, differentiates, stains for twenty to thirty minutes with saturated aqueous solution of thionin, passes up to absolute alcohol, stains for two minutes with Orange G dissolved in clove oil, and passes through xylol into balsam. Chromatin blue, chondriosomes black.

698. Formalin-Chrome Techniques.* The methods of Regaud, Bensley-Cowdry, Schridde, Murray, etc., are of importance on account of their suitability for vertebrate, and especially mammalian tissues. The tissues are fixed either in neutral formalin or in formalin-chrome mixtures, washed, and then mordanted in 3 per cent. $K_2Cr_2O_7$. As with Champy-Kull, it is possible to stain after such fixation by a variety of methods :—iron hæmatoxylin, acid fuchsin, alizarin and crystal violet, safranin, etc. The Regaud and Bensley-Cowdry methods do not preserve neutral fat in the finished sections, but by post-osmication, as for Schridde (§ 702), or Murray (§ 704), this can be done.

699. Regaud's Formol-Bichromate and Iron Hæmatoxylin (*Arch. d'Anat. micr.*, t. xi, 1910). Fix in a mixture of 3 per cent. potassium bichromate 80 volumes, commercial formalin 20 volumes, for four days, changing every day. Mordant in potassium bichromate for seven days, changing every second day. Wash in running water twenty-four hours, dehydrate (twenty-four hours each strength), clear and imbed in paraffin. Pass sections on slide down to water ; 5 per cent. iron alum at

* Note that formalin-chrome mixtures consist of a reducer and an oxidiser, and will not keep. Such solutions should always be made up just before use. Zenker's fluid, too, keeps better without the acetic acid, which, if being used, should be added just before the material is put in the fixative.

35° C. for twenty-four hours; rinse in aq. dest., not tap-water. Stain twenty-four hours in this solution:—1 grm. of pure crystals of hæmatoxylin in 10 c.c. of absolute alcohol, added to 10 c.c. of glycerin and 80 c.c. of aq. dest. Differentiate in 5 per cent. iron alum, watching process under microscope. The main point is to avoid washing out the mordant too much when the slides are being transferred from the iron alum to the hæmatoxylin. Permanent stain, very good for vertebrate tissues. See also Cowdry, *Amer. Journ. Anat.*, xix, p. 441. We find ordinary iron hæmatoxylin is quite good after Regaud fixation.

700. Kruozynski's* Mitochondrial Method. This simple method has been found useful by Miss J. C. Hill (*Arch. f. exper. Zellf.*, 1936). Fix in Cajal's fluid overnight, or a shorter period for more delicate material. Transfer to 5 per cent. bichromate for three days, wash out under tap, imbed, section in wax and stain in ordinary iron-alum hæmatoxylin.

701. Bensley-Cowdry **Acid Fuchsin and Methyl Green Stain** (Cowdry, *Contrib. Carnegie Inst. Wash.*, viii, 1918). Fix as for Regaud, either by immersion or injection; formalin should be neutralised in magnesium carbonate, and, if possible, the fixation should be done in an ice-box, but this is not necessary. Pass sections down to aq. dest. through toluol (or xylol), absolute alcohol, etc., thirty seconds in each; transfer to 1 per cent. potassium permanganate for thirty seconds, but time must be determined experimentally; then 5 per cent. oxalic acid for thirty seconds. (*Note.*—The permanganate and oxalic acid may generally be omitted.) Then rinse in several changes of aq. dest. for about one minute (incomplete washing prevents staining in acid fuchsin). Stain in Altmann's fuchsin (§ 694) as follows: dry around sections with duster, add stain, warm over spirit lamp until fumes come off; cool for six minutes; wipe around sections with duster, rinse off in aq. dest., so that the only remaining stain is in the sections (or a precipitate forms with the methyl green); pipette a little 1 per cent. methyl green over the sections for about five seconds at first, modify time as experimentally found convenient; drain off excess, plunge into 95 per cent. alcohol for a second or two. Rinse in absolute alcohol, clear in toluol, mount in balsam. Difficulties are that the methyl green may remove the fuchsin (due to incomplete chrome mordanting during fixation), or the fuchsin may have overstained (due to too much mordanting). Sometimes, if the methyl green is too weak, it is better to omit the 95 per cent. alcohol, dehydrating in absolute. The difficulties of this modification of Altmann's stain are easily overcome; we have used it for a senior histology class, and with success. Like the Champy-Kull method, this stain is not so permanent as iron hæmatoxylin. See also Bensley, *Amer. Journ. Anat.*, xii, p. 308; Duesberg, *ibid.*, xxi, p. 469.

* Compare Benoit fluid, § 707.

Acid violet may often be employed in place of methyl green, particularly in the study of the hypophysis (BAILEY, *Jour. Med. Res.*, 1920, xlii, 353). The mixture is :

Acid violet	1 grm.
10 per cent. sulphuric acid . . .	2—5 drops.
Aq. dest.	100 c.c.

The acid should be added drop by drop until the stain reaches the desired intensity.

702. SCHRIDDE'S **Method for Mitochondria, modified** (*Ergeb. Anat. u. E. Merk. Bonnet*, xx, 1911). Fix in this mixture : formol (1 part), Müller (9 parts), for two days ; then place in Müller, two to four days ; then 2 per cent. OsO_4, for two days. Wash overnight, dehydrate, clear in xylol, cut paraffin sections 5 μ. Stain as follows : iron alum hot for a quarter of an hour, then hæmatoxylin hot, a quarter of an hour. Differentiate in alum in the cold. This has the advantage over pure formol-chrome techniques in that the introduction of the OsO_4 preserves fat * ; recommended by Duesberg. With this mordanting it should be possible to stain either as for Altmann, Bensley-Cowdry, Champy-Kull, or Benda.

This method has been found by us to be one of the most useful and reliable for mammalian and bird tissues. We find it an advantage to bleach sections with 20 parts of H_2O_2 in 80 parts of absolute alcohol. The H_2O_2 must be kept in an ice safe, because if kept at room temperature it soon loses its oxygen. After bleaching, Schridde material stains well by the hot alum hæmatoxylin method.

LEVI, G. (*Arch. f. Zellf.*, Bd. xi), ovary of mammals.

10 c.c. .	2·5 per cent. $K_2Cr_2O_7$.
10 ,, .	5 per cent. sublimate containing 2 c.c. of formol.
2 ,, .	2 per cent. OsO_4.

Leave for three or four days. Wash out well in running water. Stain in Regaud, Benda, etc.

703. A. H. DREW'S **Formol-Chrome-Hæmatoxylin Method** † (*Journ. R. Micr. Soc.*, 1920). This method is used for demonstrating rod-like bodies in the cytoplasm of plant cells. These rods were, but are not now, supposed to be the homologue of the Golgi apparatus of animal cells. The method will undoubtedly be useful for studying animal tissues. 1. Fix plant root tips, etc., for twenty-four hours in a mixture of formol, 20 c.c. ; cobalt nitrate, 2 grm. ; sodium chloride, 0·8 grm. ; water to 100 c.c. (preferably at temperature of 37° C.). 2. Soak fixed tissues in gum-syrup for at least an hour, and cut sections on freezing microtome.

* See warning in § 691.

† GRASSÉ (*A. Zool. Expér.*, 1926) attributes this to Luelmo, 1923. For Protozoa you leave out steps 2 and 3.

3. Wash in water, and fix on gelatin-coated slides with formalin. See §§ 227 and 228. 4. Rinse in water to remove excess formalin, mordant at 50° to 55° C. in chromic acid 4 per cent., osmic acid 2 per cent., equal parts, on slide for varying periods—fifteen minutes to one hour, or longer. 5. Rinse in water and stain with iron alum 3 per cent. for fifteen minutes, followed by ½ per cent. hæmatoxylin for fifteen minutes, at 50° C. Differentiate in the cold in iron alum till the nuclei show pale brown. Transfer to 2 per cent. aqueous pyridin for two minutes, dehydrate and mount in xylol-balsam.

In specimens chromed for short periods the mitochondria alone are visible, while in those chromed for a longer time the mitochondria stain less well, while gradually the long Golgi elements appear in the best chromed cells. In animal cells, too, Drew finds short chroming shows the mitochondria, while it requires longer treatment in the chrome to demonstrate the Golgi apparatus. This is our own experience with the Golgi elements or "nebenkern batonettes" of Mollusca.

704. J. A. Murray's Chrome-Osmic Method for Mitochondria and Bacteria * of Mammalian Tissue. Fix tissue in formol-salt or formol-Müller overnight. Thin slices are then placed in Müller's fluid for from two to seven days, and then transferred to 2 per cent. OsO_4 for two days more. Wash overnight in running water, dehydrate, imbed in paraffin. Sections to be not more than 5 μ thick, fixed on slide, and stained in 3½ per cent. iron alum at 50° C. for fifteen minutes, followed by ½ per cent. aqueous hæmatoxylin in same way and for same time. Sections should now be jet black. If such sections be decolourised in the ordinary way in iron alum, both mitochondria and bacteria (if present) will retain the stain, and nuclei are decolourised.

If such sections are decolourised in 0·5 per cent. HCl in 70 per cent. alcohol, the mitochondria give up the lake and the bacteria remain deeply stained. At the same time the details of the nuclei are sharply stained. Wash sections for twenty minutes in tap-water, counterstain in Van Gieson, mount in balsam (Report Imp. Cancer Research Fund, 1919).

705. Double-Staining in Hæmatoxylin and Acid Fuchsin. It is well known that different cell elements have varying powers of resisting decolourisation or differentiation after iron alum or such hæmatoxylin stains. Thus in a hermaphrodite gonad or during fertilisation it is sometimes noticed that the mitochondria of the egg hold the hæmatin lake much faster than those of the sperm or spermatogenesis stages. It is possible in certain cases to make use of this fact for studying differentially cell granules, etc.

Fix tissue by some prolonged mordanting method, such as that of Champy-Kull, or Regaud. Wash out well in running water and prepare thin paraffin sections. Stain by some intense hæmatoxylin method, such as that of Benda or Heidenhain; differentiate the cell element which you wish to be stained subsequently a red colour, till it looks pale greyish under the microscope: wash well in water, and counter-

* Compare § 1332.

stain in Altmann's acid fuchsin. Extract the fuchsin to the right stage in 95 per cent. alcohol, quickly dehydrate and clear in xylol; mount in balsam. If necessary, after staining in acid fuchsin, you may apply the picric acid of Altmann's method (§ 694), but this necessitates under-differentiation in the iron alum.

We have found that after staining in the acid fuchsin you may differentiate partly in aurantia as for the Kull method (§ 695).

A method to be tried only by experienced cytologists. The difficulty is to differentiate the hæmatoxylin just to the right stage, and to avoid washing away the acid fuchsin (HANS HELD, *Arch. f. mikr. Anat.*, Bd. lxxxix).

706. On Post-Chroming and Post-Osmication in General. By soaking tissues in $K_2Cr_2O_7$, with or without CrO_3, one produces stainable compounds of cell proteins and lipoids which are not easily dissolved out by alcohol and a clearing oil. One may fix in almost any mixture not containing alcohol, chloroform and acetic acid. Wash the tissue in aq. dest. for a short time (say half an hour or less), cut into small pieces, and transfer to 3 per cent. potassium bichromate for several days, and then to 1 or 2 per cent. OsO_4 for a day. Wash under tap overnight and stain in Heidenhain or an Altmann. Thus tissues or embryos fixed in special formol-chrome, corrosive formol, chrome-corrosive, and other mixtures, which one has found most suitable for one's purpose, may be post-chromed, or post-osmicated as well. Schridde's and Murray's methods (above) include both post-chroming and post-osmication.

707. A. C. Hollande's Chondriome Method. Fix small pieces in Benoit's fluid for four hours (uranium nitrate 4 per cent. aqueous, 4 parts; potassium bichromate 5 per cent. aqueous, 6 parts; saturated mercuric chloride in saline, 5 parts, 2 per cent. OsO_4, 5 parts). After fixation place for twelve hours in a 3 to 5 per cent. solution of formalin in distilled water, then wash in ordinary water for twenty-four hours. Upgrade, toluene, paraffin. The sections on the slide are treated with xylol, then for five minutes in a mixture of equal parts of 96 per cent. alcohol and sulphuric ether containing 5 per cent. collodion, down-graded in alcohols, to distilled water. Stain in Altmann's acid fuchsin for thirty minutes; wash half to one minute in distilled water, then five minutes in an 0·5 per cent. aqueous solution of phosphomolybdic acid. Pass to a 1 per cent. aqueous solution of methylen blue for ten to twenty minutes, distilled water for fifteen seconds, then differentiate in 95 per cent. ethyl alcohol for one minute. Dehydrate in two baths of amyl alcohol for five to ten minutes, then equal parts of xylol and amyl alcohol—then xylol and balsam. Mitochondria red, protoplasm bluish-red or grey, collagen and certain cell granules deep blue, chromatin grey, bluish or rose, nucleoli bright red.

708. KOPSCH'S * **Osmium Tetroxide Method** (*Sitzungberg. d. k. preuss. Akad. d. Wiss. zu Berlin*, 1902). Osmium tetroxide solution will fix both fats and lipoids, and proteid substances. As has been mentioned above, the various cell inclusions, such as

* Referred to by some writers as the Prenant-Kopsch method.

mitochondria, Golgi apparatus, yolk and fat, are nearly always mixtures of different quantities of several definite substances, and consequently will reduce the osmic solution in varying degrees of intensity. Kopsch methods are somewhat capricious, but one gets results unequalled by other methods; for chrome-osmium, or chrome-formol, followed by iron hæmatoxylin, or Altmann generally will not demonstrate the Golgi apparatus (except in male germ-cells), while the Kopsch methods preserve and demonstrate Golgi apparatus, mitochondria, yolk, fat and chromatin structures, and occasionally neurofibrils of embryos.

For this method dissect out organs, and cut tissue into small pieces; dip these quickly into aq. dest. to remove blood or cell detritus from surface, and then transfer to a small glass-stoppered or glass-covered capsule of 2 per cent. OsO_4. Leave in a darkened cupboard for two weeks (fourteen days) at room temperature. Wash in running water for several hours, dehydrate, imbed in hard wax; section about 3 μ. Mount unstained, or stain chromatin in safranin or methyl-blue eosin. Unsaturated fats black, others yellowish, Golgi apparatus and sometimes mitochondria, black.

This method succeeds for mollusc and many invertebrate tissues, but it may be taken as superseded by the Mann-Kopsch and Kolatchew methods (§§ 710 *et seq.*). Some workers may object to the preliminary fixation in corrosive osmic of the Mann-Kopsch method, but the Kolatchew method has a preliminary fixation in Champy's fluid, and may be preferred.

709. J. Bubenaite's Method for the Golgi Apparatus. Fix for one or two days in 10 per cent. formalin. Transfer to 2·5 per cent. aqueous bichromate of potash or Müller's fluid for two days at 34° C. Rinse in 2 per cent. $AgNO_3$, and transfer to the same solution for one to two days at 34° C. Imbed and mount as usual. In our hands this produced dreadful results.

710. The Weigl or Mann-Kopsch Method (Weigl, *Bull. Acad. Scien. Cracovie*, 1912; Hirschler, *Arch. mikr. Anat.* 89). For a study of cell structure, and in general cytology, the Weigl or Mann-Kopsch method gives invaluable results. It is an alternative to the formalin-silver nitrate techniques of Cajal or Da Fano, but in addition preserves fatty substances.

The Weigl or Mann-Kopsch technique in itself is easy to work, but the subsequent steps in staining are often extremely difficult. The ordinary Kopsch technique may cause extreme shrinkage, and is not generally so specific. First fix in Mann's osmo-sublimate fluid (§ 76) for from one quarter-hour to two or three hours or more. Pieces to be fixed must be small (not exceeding a centimetre in diameter) and should only be left in the osmo-sublimate long enough to complete the penetration of the fluid. For an insect ovary, or small invertebrate, one half-hour is sufficient;

for solid tissues like nerve, longer is necessary. These times must be ascertained experimentally. After fixation the pieces are washed in two changes of distilled water for half an hour or less, according to the size of the tissue and its accessibility to the water. The pieces are transferred to a glass-stoppered bottle containing just enough 2 per cent. OsO_4 in aq. dest. to cover them. Then they are left in a cupboard at room temperature, for at least ten days, and preferably two or three weeks. Every few days the bottle should be examined to see whether the OsO_4 is evaporating, or whether it has completely disintegrated. Should either have happened the pieces should be washed quickly in aq. dest., and new OsO_4 solution added. It should be noted, however, that the osmic solution nearly always becomes slightly dark, but not until it has gone black or no longer smells of OsO_4 should new liquid be added. When the right period has elapsed the objects are taken out of the osmic.

Ludford's Modification. Experiments have shown that a much better impregnation is obtained if the reduction of the osmic acid is completed by transferring the pieces of tissue to water kept at 38° C. for one or two days before washing in running water preparatory to transference to alcohol (50 per cent.) (see § 714). They are then upgraded and imbedded in hard paraffin. Sections to be cut from 3 to 6 μ. They are stuck on the slide with albumen and water in the usual way and dried overnight. One of the slides is taken, the wax removed in xylol, and it is mounted in xylol balsam. Examination of this slide will enable one to ascertain to what extent the process has acted successfully. In completely successful preparations the Golgi apparatus, yolk and fat alone are blackened, while nuclear organs, mitochondria and cytoplasm are stained in shades of yellow and greenish-brown, Having studied this untreated slide, and noted the extent of the blackening effect of the OsO_4, one may then proceed to make experiments. Several alternative methods may be tried :—

(*a*) The blackening may be extracted step by step in turpentine, and the appearance of the cell granules studied at intervals. An alcoholic solution of hydrogen peroxide can often be used with advantage for this purpose (20 per cent. of hydrogen peroxide added to 80 per cent. alcohol).

(*b*) If the mitochondria are not stained black by the OsO_4, one may proceed directly to Altmann's method (see § 694).

(*c*) The nuclear structures may be stained in safranin, crystal violet, or acid fuchsin. The sections are brought down to distilled water and transferred to watery solutions of the dye. A few minutes generally suffice to stain the nuclei.

LUDFORD (*Jour. R.M.S.*, 1925, pp. 31—36) recommends the employment of neutral red as a counterstain. Sections which have not been bleached are brought down to water. They are then stained for half a minute, or less, in a dilute solution of neutral red (1 grm. of neutral red in 1000 c.c. of distilled water, with 2 c.c. of a 1 per cent.

solution of glacial acetic acid). The excess of stain is then rinsed off the slide with distilled water. After shaking off the distilled water, absolute alcohol is poured on the sections. When the correct degree of differentiation has been attained it is cleared in xylol and mounted in balsam.

We find that in successful Mann-Kopsch preparations, especially of Invertebrata, the mitochondria do not generally become black, but are either unstained or go yellowish. In many, but not all, cases it will be found that where the mitochondria do become black after OsO_4, colour is more readily extracted from them than from the Golgi elements, so that a distinction can nearly always be made by the Mann-Kopsch method itself, without recourse to other methods which will generally stain mitochondria and not Golgi apparatus (Regaud, Flemming, as described in § 693). Among the most useful differentiation or extraction methods after Mann-Kopsch, *turpentine is probably the best.* The wax is removed from the sections on the slide by means of xylol, and the slide is transferred to a jar of turpentine. After about half a minute the section is examined under $\frac{1}{6}$th inch objective, and the effect of the turpentine is noted; one sometimes finds that the black colour in fat globules and yolk spheres is extracted before a quarter of an hour has elapsed, while the Golgi apparatus retains its black condition. In most cases it is therefore possible to distinguish between yolk and fat on the one hand, and the Golgi apparatus on the other.

711. Mann-Kopsch-Altmann Combination (GATENBY, *Journ. Roy. Micr. Soc.*, 1921). If examination of the first Mann-Kopsch section showed that the Golgi apparatus was blackened, and the mitochondria were either not stained or only straw or light-brown coloured, one may proceed directly to the Altmann stain. Should the examination show that the mitochondria as well as the Golgi apparatus have become blackened, the sections must be extracted in turpentine in an endeavour to remove the blackening from the mitochondria. If the latter treatment does not succeed properly the only course is to make new Mann-Kopsch preparations, allowing less time in the OsO_4, say seven or eight days instead of the two weeks.

When one has succeeded in procuring sections in which the Golgi apparatus alone is blackened, it is possible to stain in Altmann's anilin acid fuchsin and picric acid method, so that the mitochondria (and nucleoli) become red, the Golgi apparatus is black and the ground cytoplasm yellowish. The Mann-Kopsch sections are brought down to distilled water, and cautiously treated in a 0·5 to 0·125 per cent. solution of potassium permanganate. They should be watched under the microscope. As soon as the cytoplasm of the cells has become dark brown in colour, and before any marked change in the impregnation of the Golgi apparatus occurs, the slides are washed rapidly in distilled water, and treated with sulphurous acid—about 5 to 10 per cent. solution. This bleaches the cytoplasm almost instantly, while the Golgi apparatus remains black. The slides should then be washed thoroughly in running water and stained (§ 694).

The following modification in staining has been used with success. After bleaching sections, and thoroughly washing, Altmann's anilin acid fuchsin is poured on the slides, which are heated until steaming occurs. They are then allowed to stand for half an hour. The excess stain is finally washed off with distilled water, and the slides are brought direct to absolute alcohol in which they are differentiated (LUDFORD, *Jour. R.M.S.*, 1926, pp. 107—109).

In a series of experiments carried out with tumour cells it was found that the length of time a tissue remains in the Mann's fluid has practically no effect upon the subsequent impregnation of the Golgi apparatus with osmic acid (LUDFORD, *Jour. R.M.S.*, 1924, pp. 269—280). When the pieces have remained for longer periods in Mann's fluid, however, sublimate crystals may be formed in the tissues. These can be removed by treating the sections with Lugol's solution.

The Mann-Kopsch technique can be used in combination with the Kull staining method (§ 695). We find that the cells are rather liable to overstrain in the toluidin blue, which must be left on for a very short time.

Explanation. Mann's osmo-sublimate fixes the cells successfully, because the $HgCl_2$ aids penetration and the OsO_4 is not so concentrated as to cause shrinkage. Thus, before the tissue is transferred to the Kopsch fluid (OsO_4 of 2 per cent.), a complete fixation has taken place and the distorting effect of the strong OsO_4 is avoided. Left for two weeks, the lipoid materials which partly form the substance of the Golgi apparatus, the unsaturated fats, and the special lipoids of the mitochondria, are all able to reduce the OsO_4 in varying degrees. The subsequent treatment of the sections by turpentine (oxidiser) introduces a further differentiation, and so the various inclusions can be distinguished. The acid fuchsin stains presumably the lipoid content of the mitochondria.

712. Shorter Osmication Methods. The length of time taken by osmic acid to penetrate tissues and impregnate the Golgi apparatus has led to various modifications of the Kopsch method being employed to bring about the result in a shorter time. Experiments have shown that reduction of the osmic acid is considerably hastened by a moderate rise of temperature. In well-fixed material, however, there are considerable secondary changes in the cytoplasm on incubation in watery media, at temperatures ranging from 40° to 60° C. (LUDFORD, *Jour.R.M.S.*, 1924, pp. 269—280). This fact renders it undesirable to employ temperatures much above 35° C.

Of the methods in which hot osmic acid is employed the following have been found to give excellent results :—

713. The Kolatchew Method as Modified by Nassonow. The material is fixed in Champy's fluid to each 10 c.c. of which 2 or

3 drops of a 0·1 per cent. of pyrogallic acid is added, or else in a modified Champy fluid. The following is recommended :—

6 per cent. potassium bichromate . .	2 c.c.
1 per cent. chromic acid	2 ,,
2 per cent. osmic acid	2 ,,

Various other modifications have also been employed (see Nassonow, *Archiv. f. mikr. Anat.*, 1923, 97, 136—186, and 1924, 103, 437—482; also Weiner, *Archiv. Russes d'Anat. d'Hist. et d'Embry.*, iv. 1925, 37—164). After twenty-four hours the material is thoroughly washed (overnight), and then transferred to 1 to 2 per cent. osmic acid at 30° to 35° C. for three to seven days. Nassonow suggests the following method for determining when the correct degree of impregnation is attained. Small pieces of the tissue are broken off from the fragment in the warm osmic acid. They are washed, and then mounted in glycerin. By pressing on the coverslip the particles can be disintegrated, owing to their brittle nature. In this way individual cells can be examined under the microscope. When the material is found to be suitably impregnated the fragments are washed in running water, upgraded through the alcohols and imbedded in wax in the usual manner.

714. Ludford's Modified Mann-Kopsch Method. The tissue is fixed in Mann's corrosive osmic fluid for eighteen hours. It is then washed in distilled water for thirty minutes. Osmication is carried out in an incubator at 30° C., using 2 per cent. osmic acid, for three to five days, depending upon the nature of the material. The reduction process is completed by leaving the tissue for another day in water at 30° C. The material is then brought up through the alcohols and imbedded in paraffin wax (*Jour. R.M.S.*, 1926, 107—109). Sections should be counterstained as described § 710, page 314.

715. Strength of Osmic Solution. Kolatchew used 1 or 2 per cent. osmic acid, while other workers including Nassonow, Kopsch, Morelle and Deineka all used 1 per cent. We have nearly always used 2 per cent., but there has been a tendency nowadays to use 1 per cent., which in view of the cost of osmium tetroxide is more economical.

716. On Counter-Staining Weigl or Kolatchew Sections in Iron-Alum Hæmatoxylin or Acid Fuchsin. The possibility of doing this successfully should be noticed (see Gatenby and Beams, *Q.J.M.S.*, 1936). The advantage is that one can often get both categories of inclusions stained in different colours. For example, in spermatogenesis the Golgi apparatus will be black with osmic acid, and the mitochondria blue with hæmatoxylin or red with acid fuchsin. This is sometimes very useful. The main difficulty is to hit off the time of bleaching properly. No exact times can

be given. The wax should be thoroughly removed from the slides, which are brought down to distilled water. Permanganate of potash (§ 711) is then poured on the sections and they are examined under the $\frac{1}{3}$ or $\frac{1}{6}$ objective to make sure that the cells are saturated evenly. The oxalic acid is then added, and the slides again washed in distilled water for a few minutes. They should now stain like ordinary chrome-osmium (Champy) slides, in alum hæmatoxylin (all day in alum, overnight in hæmatoxylin) or by Altmann's acid fuchsin (§ 694).

717. **Osmic Vapour Method** (W. CRAMER, *Imp. Cancer Research Fund Report,* 1919). Choose a small glass-stoppered bottle, and place a piece of wide glass tube open at both ends, in the bottom. Arrange a piece of gauze over the top of the inner tube. Add some 2 per cent. OsO_4 to the outer vessel. Objects to be fixed by the osmic vapour are placed on the gauze. All the surrounding (fatty) tissue should be removed from the organ or material to be treated ; if too dry the outside of the material should be slightly wetted.

The bottle, with object suspended in the OsO_4 vapour, is kept at a temperature of 40° C. for one and a half hours. Removed from bottle, the tissue is placed in 50 per cent. alcohol and upgraded and imbedded in paraffin. Cut sections, mount in balsam without staining. Such wax sections may be treated in 10 per cent. hydrogen peroxide in 80 per cent. alcohol for two hours, after which they may be stained in ordinary methods (*e.g.* iron hæmatoxylin).

This method is employed by Cramer for the demonstration of adrenalin in cells of the adrenal glands.

GATENBY (*Quart. Journ. Micr. Sci.,* 1920) suggests two modifications.

(*a*) Fix as above for one and a half hours, and then transfer to 2 per cent. OsO_4 in water at 37° C. for several days as for Kopsch. Then wash in water for several hours, dehydrate, imbed and section. Mount unstained, or cautiously treat in permanganate of potash or hydrogen peroxide and then stain in acid fuchsin (Altmann) or iron hæmatoxylin.

(*b*) Tissues may also be fixed as above, and then transferred to Altmann's or Champy's fluid, and subsequently stained in Altmann's fuchsin and picric acid.

Cramer fixes wet films for about three minutes. We think that a subsequent treatment of films as in above two paragraphs should be useful. The main point to note is that substances in a tissue which might be dissolved out or altered by the water added to the OsO_4 crystals are fixed *in situ,* and without the danger of alteration. This method should be of value to histologists and cytologists.

718. SJÖVALL'S **Formol Osmic Acid Method*** (*Anat. Hefte,* Bd. xxx, 1906). Superseded by Weigl's method, *op. cit.*

Material fixed in formalin, but without chrome salts or platinum chloride, may be used for Sjövall's technique (*Anat. Hefte,* Bd. xxx). Fix pieces of tissue or small embryos in neutral formalin (5 to 20 per cent. neutralised with magnesium carbonate) for two days. Cut into smaller pieces and wash in several changes of aq. dest.

Transfer to 2 per cent. OsO_4 solution for from two to fourteen days at room temperature, as for Kopsch. Wash well in water, dehydrate clear and imbed. Cut sections 3 μ, if necessary decolourise in peroxide (Solger, § 613) and mount unstained in balsam.

719. General Remarks on the Silver Nitrate Golgi Apparatus Methods. These methods all suffer from the disadvantages of

* Still the only osmic method possible for formalin fixed human material

simple formalin fixation. Such fixation often gives delightful results for such things as mammalian liver and brain, but poor results for small invertebrates like crustaceans or worms. For such animals Aoyama's fixative is the least bad, and we have had some very fine results on *Saccocirrus* with Da Fano's fluid. On the other hand, it is almost impossible to do any good with animals like *Daphnia* or *Cyclops*. The finished sections are shrunken and generally useless. There seems little doubt that the osmic methods used, such as those of Nassonow and Weigl, are the only reliable ones for most small invertebrates.

Many Da Fano * and Cajal preparations we have seen have been made carelessly, and the sections have been filled with granular precipitates of silver. This makes the material unreliable and useless for research; such a fault is due to excess silver left before the reduction stage. *It has been concluded by a number of workers that too long immersion in the fixative causes the mitochondria as well as the Golgi apparatus to become impregnated. It is recommended to cut down the fixation time as much as possible and to make the pieces small. In such cases fixation will be complete in one to two hours.*

Results may be improved by fixing tissue in chilled Da Fano or Cajal, and the worker is strongly recommended to try the cold method as well as that at room temperature.

720. The Supposed Chemical Basis of the Formalin Silver Nitrate Techniques. A number of workers have noted that these methods correspond somewhat to several techniques of doubtful specificity used microchemically to demonstrate chlorides.

721. GOLGI-VERATTI'S **Method** (see GOLGI, *Anat. Anz. Verh. Anat., Ges.*, xiv, 1900, p. 174). Small pieces are hardened for a time varying from a few hours to ten days or longer in Veratti's mixture, consisting of—

5 per cent. potassium bichromate . .	3 parts.
0·1 per cent. chloroplatinic acid	3 ,,
1 per cent. osmic acid	3 ,,

From time to time pieces are put in one or other of Golgi's rejuvenating fluids (as described in § 1035), and thence into 0·8 to 1 per cent. silver nitrate. Sections are cut and mounted as by Golgi's bichromate and nitrate of silver method (see § 1036).

722. GOLGI'S **Arsenious Acid and Silver Nitrate Method** (*Arch. Ital. Biol.*, xlix, 1908, p. 272). Small pieces of quite fresh tissues are fixed for three, six, eight or twelve hours in equal parts of 20 per cent. formalin, saturated solution of arsenious acid, and 96 per cent. alcohol. After a quick wash with distilled water, they are passed for some hours (or days) into 1 per cent. silver nitrate, and then treated with a reducing fluid, usually Cajal's hydroquinone mixture (hydroquinone 20 grm., sodium sulphite 5 grm., formalin 50 c.c., water 1000 c.c.). Wash quickly, dehydrate, and imbed either in celloidin or paraffin. The sections are toned with equal parts of 1 per cent. gold chloride and a

* Da Fano preparations should be cleared in cedar wood oil, as xylol shrinks them badly.

mixture consisting of water 1000 c.c., with 30 grm. each of sodium hyposulphite and ammonium sulphocyanide, and then rapidly bleached by the following method, due to VERATTI :—Wash the toned sections in distilled water and transfer them for one, two or three minutes into potassium permanganate 0·5 grm., distilled water 1000 c.c., sulphuric acid 1 c.c. ; wash again ; transfer into 1 per cent. oxalic acid until the yellowish colour imparted to the sections by the potassium permanganate has disappeared ; wash thoroughly in repeatedly changed distilled water ; counterstain, dehydrate, and mount as usual.

723. RAMÓN Y CAJAL'S **Uranium Nitrate and Silver Nitrate Method** (*Trab. Lab. Invest. Biol.*, Madrid, xii, 1914, p. 127). (1) Small pieces of quite fresh tissues are fixed for ten to fourteen hours in a mixture of neutralised formalin 15 c.c., distilled water 85 c.c., uranium nitrate 1 grm. Instead of uranium nitrate, uranium acetate, as suggested by Del Rio-Hortega, may be sometimes used. Should a very fine reaction be desirable, the following formula may be employed :—Uranium nitrate 1 grm., ethyl or methyl alcohol 30 c.c., distilled water 80 c.c. neutralised formalin 15 to 20 c.c. (2) After a quick wash in distilled water pieces are transferred into 1·5 per cent. silver nitrate and kept therein for thirty-six to forty-eight hours at room temperature. If the pieces are only a few and small 1 per cent. silver nitrate will be sufficient. (3) Wash quickly and reduce for eight to twenty-four hours in hydroquinone 1 to 2 grm. formalin 15 c.c., distilled water 100 c.c., sodium sulphite 0·5 grm. (4) Wash quickly, imbed in paraffin or celloidin, or make sections by the freezing method. (5) Tone and counterstain sections if desirable. Dehydrate and mount as usual.

Best results are obtained from vertebrates, preferably kittens and young rabbits. The method may be applied to human material, if available in a sufficiently fresh condition. With invertebrates results are not so good, and rather uncertain, so that Cajal advises a simple fixation in formalin or formalin-acetone, followed by impregnation with silver nitrate, as by his reduced silver methods for neurofibrils.

724. DA FANO'S **Cobalt Nitrate Modification** (*Proc. Physiol. Soc. Journ. Physiol.*, liii, 1920 ; *Journ. R. Micr. Soc.*, 1920, p. 157). Small pieces of quite fresh tissues are fixed for six to eight hours at room temperature in cobalt nitrate 1 grm., distilled water 100 c.c., formalin 15 c.c. The solution can be prepared beforehand, and keeps unaltered for months. The formalin need not be neutralised unless strongly acid or containing free sulphuric acid, in which case it is necessary to neutralise it by one of the usual methods. For the fixation of embryonic organs and in all cases in which a shrinkage of delicate tissues is to be feared, the quantity of the formalin may be reduced to 10, 8, or 6 c.c. for every 100 c.c. of distilled water. The time of fixation should be shortened to three to four hours or even less in the case of very small pieces, such as

spinal ganglia of mice and rats, the pituitary body of the same animals, etc. Pieces of spinal cord, cerebrum, cerebellum of adult animals give better results if fixed for about eight to ten hours. The fixation may be prolonged in special cases to twelve to twenty hours, but should not exceed twenty-four hours. The fixation in an incubator at a temperature varying between 25° and 37° C. has been attempted with success in the case of tissues of adult subjects, but it leads to a staining of both the internal apparatus and intracellular formations, which, according to their morphology are to be considered as mitochondria.

For the impregnation, Da Fano quickly washes the pieces in distilled water, makes their surfaces smooth if necessary, and then places them into 1·5 per cent. silver nitrate in the dark for twenty-four to forty-eight hours at room temperature. For very small fragments, 1 per cent. silver nitrate may be used, whilst for pieces of spinal cord of adult subjects, 2 per cent. should be preferred. For the reduction he uses Cajal's hydroquinone-formalin mixture, taking care in further recutting the pieces before transferring them into the reducing fluid, so that their thickness should not exceed 2 mm. He dehydrates, clears in cedar oil and imbeds pieces, preferably in paraffin, or he makes sections by the freezing method. He usually tones these by means of 0·2 per cent. gold chloride, fixes with 5 per cent. sodium hyposulphite, counterstains and mounts as usual.

The method gives good results also with material from lower vertebrates and invertebrates.

725. Cadmium Chloride Formol Golgi Apparatus Method (F. Aoyama, *Zeit. f. wiss. Mikr.*, 1930). Fix small pieces of tissue in cadmium chloride 1 c.c., formol, neutral 15 c.c., distilled water 85 c.c., for three or four hours. Rinse quickly in two changes of distilled water, and transfer to 1·5 per cent solution of silver nitrate for ten to fifteen hours at 22° C. Rinse quickly in two changes of distilled water, preferably in a dark room, and transfer for five to ten hours to the reducing solution (hydroquinone 1 part, neutral formol 15 c.c., distilled water 85 c.c., 0·1 to 0·15 gm. of sodium sulphite, sufficient to produce a yellowish tinge). Wash thoroughly in tap water, upgrade, imbed and section. The sections may be counterstained in carmine or hæmatoxylin and eosin. Cold-blooded animals need longer fixation and impregnation than warm-blooded, for which the above given times are suitable.

After having seen preparations of various vertebrate and invertebrate tissues made by this method, we have concluded that it is the best of the three silver formalin methods, and the nearest approach to a good osmic Golgi technique. *It is reliable, remarkably specific, and causes less incrustation of dictyosomes than other silver methods.*

726. Other Methods and Modifications. Besta (*Anat. Anz.*, xxxvi, 1910, p. 477) fixes for two days in 20 parts of formol with 2 of acetic aldehyde and 80 of water, washes for twenty-four hours in distilled water changed seven or eight times, and puts for two days in 4 per cent. solution of ammonium molybdate, makes paraffin sections, stains in a 1 : 1000 solution of thionin, differentiates in 3 parts of creosote to 1 of absolute alcohol, and passes through pure creosote and xylol into neutral balsam. Recommended for Purkinje cells and spinal ganglia of young animals.

Suchanow (*Neurol. Centrbl.*, xxi, 1902, p. 777) has obtained good results by the use of Golgi-Veratti mixture, keeping pieces of spinal cord and spinal ganglia for twenty to thirty days in the mixture and for two to three days in the rejuvenating fluid.

Legendre (*Anat. Anz.*, xxxvi, 1910, p. 209) omits the toning and bleaching by Golgi's arsenious acid method, and imbeds in paraffin.

Similarly Collin et Lucien, *Bibliogr. Anat. Supp.*, 1909, p. 238.

Savagnone (*Pathologica*, i, 1909) silvers pieces fixed in Golgi's arsenious acid mixture with 30 c.c. of tachiol (10 per cent. silver fluoride) in 100 of water.

Carleton (*Journ. R. Micr. Soc.*, 1919, p. 321) reduces pieces treated according to Cajal's uranium nitrate method for only two hours in the usual hydroquinone mixture.

Penfield (*Brain*, xliii, 1920) has successfully employed Cajal's uranium nitrate method for his experimental investigations on the alterations of Golgi's apparatus in nerve cells of spinal cord and spinal ganglia of young cats. He adds 20 c.c. formalin (instead of 15) to Cajal's fixing fluid and as much as 1·5 grm. of sodium sulphite to the hydroquinone-formalin solution. He finds it imperative to dehydrate pieces very quickly before imbedding them in paraffin. In order to obtain perfect fixation of the spinal cord he sometimes performs a laminectomy in the lower lumbar region of the anæsthetised animal, passes a needle in the subarachnoid space, and allows the fixative to flow in "under a gravity pressure of 75 cm." The heart stops about a minute after the beginning of the injection, which is continued for twenty hours. At the end of this time the cord is removed, pieces cut and dropped directly into the silver bath.

727. Counterstaining. Penfield finds it particularly useful to immerse untoned sections into a diluted solution of Unna's polychrome-methylen blue for one to four hours, this being followed by passage through alcohols of increasing strength and differentiation in absolute alcohol.

By this method, also Holmgren's trophospongium is sometimes stained. But for the study of the relationship between the latter and Golgi's apparatus, Penfield (*in litteris*) prefers to make drawings of the apparatus from certain selected cells, subsequently removing the coverslip and bringing the slides through graded alcohols into 5 per cent. iron alum for twelve to twenty-four hours. This removes all silver from the cells as well as the counterstain, and at the same time mordants the tissues for further staining by Heidenhain's iron hæmatoxylin method. If the proper amount of differentiation has been secured of the particular cells already drawn, the trophospongium will be found stained with great detail.

Counterstaining may also be done in borax carmine (three to twenty-four hours) 0·5 neutral red or toluidin blue in water

(Da Fano), differentiating in 95 per cent. alcohol, safranin O. (Grübler) saturated in equal parts of absolute alcohol and anilin oil water, fifteen minutes to several hours, differentiating in 90 per cent. alcohol. We have constantly used Mann's methyl blue eosin in this laboratory. If iron-alum hæmatoxylin is to be used, the alum bath must be short or the silver will be extracted, but Bowen (*Anat. Rec.*, 1928) says that if the sections be toned, see § 724, the impregnation is unaffected. Bowen believes that this double method has great possibilities.

Of all these methods Mann's methyl blue eosin is the easiest and best, as this fine stain usually acts normally on formalin material.

728. On Toning Blue with Ferric Salts. In this laboratory J. S. Barlee has used the following method to tone formalin silver nitrate slides. The result is a blue preparation which has certain possibilities—*e.g.* to discriminate between pigment and Golgi apparatus. Solution A. Potassium ferricyanide 1·56 grm. H_2SO_4 concentrated 0·3 c.c., aq. dest. 1000 c.c. Solution B. Ferric ammonia citrate 1·56 grm. H_2SO_4 concentrated 3 c.c., aq. dest. 1000 c.c. Mix equal portions of A and B at time of use. Bring slides to distilled water, add solution for about fifteen minutes. Wash slides for at least ten minutes under tap after toning. The stock solutions do not keep well.

729. Difficulties and Faults in Formalin Silver Methods. Complete failure to impregnate the Golgi apparatus sometimes occurs. This may be due to bad or old formalin—commercial museum formalin is useless. It may be due to the silver solution being too old. Unless the previous trials have been successful, it is better to make up new silver nitrate. Keep packets of silver nitrate weighed up, so that the proper quantity can be placed in water when new solution is needed. Reduction is naturally a vital part of the method, one must see that this fluid is right. The colour of quite efficient reducing solutions may vary from light to dark sherry. Faults may be traced to the washing out after fixation, and after silver nitrate. If one washes out too much naturally the methods will not succeed, but this fault can be guarded against by having a number of pieces of different sizes.

Granulated preparations are usually due either to using too strong silver nitrate, or to not washing out enough before reduction. Again, pieces of different thickness will help to solve the difficulty.

730. E. Vazquez Lopez Mixed Osmic-Silver Method for the Golgi Apparatus (of Tissue Cultures) (*Arch. Espan. Oncologia,* tomo III, 1932–33).

Fix in osmic vapour for several minutes until the object becomes opaque through the action of the vapour. Then wash well in distilled water and submerge in 3 per cent. tannic acid for twenty-four hours at room temperature. Transfer to a petrie dish with one drop of ammonia. (Then place in Rio Hortega's ammoniacal silver reagent, prepared by adding to 30 c.c. of 10 per cent. silver nitrate, forty drops of 40 per cent. caustic soda. The precipitate is allowed to settle, the supernatant

liquid decanted, and the precipitate is washed ten or twelve times in about a litre of distilled water; 50 c.c. of water are then added to it, and then ammonia drop by drop until the precipitate is dissolved. This stock is brought up to 150 c.c. by adding distilled water, and keeps for months.)

Impregnation is carried out in three dishes, the first containing 10, the second 15, the third 20 drops of the above solution in 10 c.c. of distilled water. The objects are left in each strength until the silver begins to precipitate, making the liquid turbulent. In the last dish there should be no precipitation.

Wash in distilled water. Transfer to gold chloride. Fix in sod. hyposulphite 1 per cent. for a short time only. Wash in distilled water, upgrade and mount in balsam. In our hands results no better than Weigl.

730a. Combined Golgi Apparatus and Fat Stain (GATENBY, *Biological Technique*, 1937). A successful method is the combined Aoyama (or Da Fano) and Sudan IV. You prepare either smears or frozen sections by, preferably, the Aoyama method. The piece of tissue cannot be imbedded in wax as the upgrading in alcohol to xylol removes the fat, so frozen sections must be cut, and the thinner the better, or the material may be teased. After the Aoyama smear, or the block of tissue has been brought into reducing fluid as described in § 725, it should be washed in water. The smear goes immediately on to the fat stain, whereas the block of tissue is sectioned, and then stained in Sudan IV, or a small piece is first stained in Sudan IV and then teased up in glycerin and water (1 : 1). In both cases the mountant is Berlese (§ 1188) and not glycerin, glycerin jelly, or Farrants, the reason being that glycerin attacks the silver.

Exactly the same can be done after the Weigl or Kolatchew methods. You stain in Sudan IV and mount in Farrants, glycerin or glycerin jelly (§ 691).

In both the silver nitrate and the osmic method the Golgi apparatus is black, and fat is red, but in some osmic preparations the colour of the fat is brownish red rather than the bright red of the formalin silver preparations.

731. Plan for Material Fixed in One, Two or Three Lots.

A. (1) Fix in Cajal or Aoyama

↙	↓	↘
(2) Continue on with Golgi apparatus method. ↓ (3) Counterstain in Mann's methyl blue eosin.	(2a) Post - chrome in 3 per cent. to 5 per cent. $K_2Cr_2O_7$ for three days. ↓ Wash out under tap overnight. (3a) Stain in iron hæmatoxylin or acid fuchsin for mitochondria.	(2b) Wash out, cut frozen sections or tease, for fat stains, mount in Berlese, etc. Also chlorocarmine.

B. (1) Fix in Bouin-Allen

(2a) Continue on for chromosome and chromatin study.	(2b) Stain for glycogen.	(2c) Use general histological stains.

C. (1) Fix in Champy or Altmann

(2a) Continue on for mitochondria by Altmann or iron hæmatoxylin.	(2b) Wash out thoroughly, proceed to Kolatchew for Golgi bodies.	(2c) Cut frozen sections or tease, mount in Farrant for fats

The above is the plan used in this laboratory for getting material from hospitals, abattoirs, and from a distance. Plan A is cheap and needs only the provision of a Winchester of fixative. From it a great deal can be done. Plans B and C are an added help, but not necessary if the preparations in A are successful. So far as cytoplasmic bodies are concerned it is usually possible to make good preparations even after the pieces have been several days, or even a week, in fresh fixatives mentioned.

732. The Beams' Ultra-Centrifuge in the Study of Cell Inclusions. This is an air-driven centrifuge, consisting of a hollow top with a screw cap. This top spins on a column of air produced by an air pump of the type used for tyre inflating in garages. Recently this centrifuge has become an article of commerce, and our experience with it has convinced us that it is quite indispensable for cytologists and protozoologists. No study of granules in oögenesis, or in such organisms as amœbæ or flagellates, can be considered satisfactory unless recourse has been made to material ultra-centrifuged.

In an experimental ultra-centrifuge made in this laboratory by R. Brown, it was found that even with only two holes in the stator crater, the centrifuge worked well. Gatenby * gives sufficient details for constructing a small ultra-centrifuge, which any good mechanic could turn out on a lathe. The holes in the crater of the stator must be staggered so as to make the rotor revolve. The centrifuge must be placed inside a box, lined with sand for safety, should the rotor disintegrate at high speed.

733. Parat's Techniques for the "Vacuome." Bhattacharya (*Allahabad Univ. Studies,* 1927–28) describes Parat's various techniques as follows :—

For vital staining, it is essential that a very good quality of neutral red is used. 1/500 neutral red (Krall) in 6/1000 physiological salt solution, in proportion of 1 to 500 volumes respectively, is placed in bath

* "Biological Laboratory Technique," Churchill, 1937. Centrifuge in neutral salt gum, § 781 and § 1430.

at 38° C. for twelve hours or so. The above should be mixed before use with an equal part of Janus green 1/500 in 6/1000 salt solution. Sea water dissolves Janus green equally well. According to Bhattacharya, Janus green by itself is not a good specific either for " Golgi bodies " or even for the detection of mitochondria, for after a quarter of an hour it becomes harmful and causes vesiculisation and fragmentation of the chondriome. By prolonged action it can colour the " Vacuome " also, and thus confusion between the two structures is caused. Soon after the " vacuome " is coloured by neutral red, Janus green can be introduced under the cover slip cautiously. Generally within a quarter of an hour, sometimes after prolonged treatment, mitochondria make their appearance. They do not, however, last long.

Fixatives used :—

(1) Nassonow-Champy modification by Parat and Painlévé—

$K_2Cr_2O_7$	3 per cent.	(2 vols.)
Acid Chromic	1 ,,	(2 vols.)
Acid Osmic.	2 ,,	(1 vol.)

Fix for six to twenty-four hours ; wash in water eighteen to twenty-four hours ; transfer to osmic acid 2 per cent. for twelve days ; sections to be cut 3—5 μ thick. Bleaching by Henneguy's method ; staining by Altmann-Kull technique.

Henneguy's bleaching method is very useful for removing the blackness of over-osmicated material. It consists in washing slides rapidly before staining in a 1 per cent. aqueous solution of potassium permanganate, and afterwards in a 4 per cent. solution of oxalic acid. The bleaching should be controlled under the microscope. Sometimes over-osmication is an advantage, for in certain cases " Golgi bodies " take on their characteristic black colour long after the fat bodies have been blackened.

(2) Dietrich after Parat and Bergeot.

Fix in Regaud's fluid (bichromate-formol) or better still in Helly's fluid for twenty-four hours ; afterwards in a saturated solution of potassium bichromate at 38° C. in the bath for forty-eight hours ; wash in water for twelve hours ; stain in hæmatoxylin.

Hæmatoxylin in a little alcohol	1 gr.
Solution acid acetic 2 per cent. in water. . .	100 c.c.

Differentiate in potassium ferricyanide solution of Weigert. According to Parat this method gives a negative result for " Golgi bodies," for their presence is indicated only by vacuoles, whereas lipoids such as mitochondria and yolk are stained black.

734. Differentiation between Cell Inclusions. It is frequently somewhat difficult to distinguish between the various categories of cell inclusions. In this section we have provided a series of tables intended to act as a tentative guide to the interpretation of the various images got by representative cytological techniques. These tables are based on work carried out on animals of most orders, *but it would be injudicious for the researcher to depend upon them implicitly*, because many exceptions are met with, and the personal factor is to be taken into consideration. The use of such tables, if made with several methods and in conjunction with a

careful study of the origin and morphology of any doubtful cell body, will, however, provide reliable evidence for identification. Another warning must be given—never try to ascertain the nature of granules in developing eggs without first studying the oögenesis of the animal in question. Eggs after spawning or laying are difficult objects to study by these methods, and even the most experienced worker is unable to give an interpretation until he has worked at the oögenesis. It should also be remembered that there are periods in the development of the cell during which the mitochondria are often able to resist becoming dissolved in lipoid solvents; these periods are in the early spermatogonium in some animals, and during the last stages of spermatogenesis (spermateleosis) in all animals, and sometimes in large oöcytes. See also the work of REGAUD, *Arch. d'Anat. micr.*, xi.

Nota Bene. With regard to the oil used for clearing and imbedding, it should be pointed out that all these tables are based on preparations cleared and imbedded in xylol, which occasionally tends to extract lightly osmicated fat. Vegetable oils like cedar wood oil seem to be less active in this way. We have rarely * found that chrome-osmicated fat, or "Kopsched" fat, is extracted either by xylol or xylol-balsam. See also § 651.

735. Differentiation between Mitochondria and Fat (Olein, Stearin and Palmitin Mixtures especially).

Method employed.	Fresh tissue stained in Herxheimer's scarlet red or Sudan IV.	Fixed in Flemming-with-acetic acid examined in unstained sections on slide.	Kopsch or Mann-Kopsch.	Regaud or formalin fixation, iron hæmatoxylin.	Fresh tissue stained in Janus green 1 : 10,000.
Mitochondria.	Will not stain brightly, generally dissolved away.	Do not show, generally dissolved away, except in certain cases where the mitochondria are more resistant to acetic acid.	Yellow or black; if black, colour often difficult to extract in turpentine; if yellow, can be stained in acid fuchsin of Altmann.	Black.	Green.
Fat.	Stains brightly. (It should be noted here that while Herxheimer's scarlet red will not stain mitochondria it may possibly stain lipoids other than true fat.)	Black.	Black: colour easily extracted after a few hours in turpentine.	Not stained, as it has been dissolved out by the clearing reagent (xylol or chloroform, not vegetable oils).	Does not stain.

* This certainly happens in the case of the delicate fat vacuoles of tissue-cultured fibroblasts, see § 691.

736. Differentiation between Golgi Apparatus and Mitochondria.

	1.	2.	3.	4.	5.
Method employed.	Formol-silver nitrate methods of Golgi, Cajal, Da Fano.	Kopsch, Mann-Kopsch (osmium tetroxide methods).	Flemming-without-acetic, Regaud, Champy, formalin, etc., followed by iron-alum hæmatoxylin.	Janus green 1 : 10,000.	Ultra-Centrifuge.
Golgi apparatus (dictyosomes, nebenkern batonettes, idiozome rods, etc.).	Black (even when untoned).	Black.	Does not show except in oögonia or very young oöcytes, and in male germ cells: rarely in other cells. When stained generally less intense than the mitochondria. In some cases a negative picture of the Golgi apparatus is obtained.	Rarely stains (except in male germ cells).	Passes centripetally.
Mitochondria	Golden (untoned) greyish (toned), more rarely black.	Not stained, yellowish, or more rarely black.	Stain black, or dark grey.	Green.	Pass centrifugally.

737. Cytoplasmic Inclusions in Gametogenesis. In the table below is a summary of the fixing and staining reactions of the inclusions during oögenesis and spermatogenesis. In the male germ cells the Golgi apparatus (nebenkern bodies) show throughout; those of the egg can generally only be demonstrated by methods 1 and 2. If yolk granules contain olein or such unsaturated fat they will stain in OsO_4 like fat, but by slow decolorisation as in paragraph 2 (with turpentine) their proteid basis will be noted and they will generally be demonstrated in methods 1 and 4, while fat vacuoles disappear completely. See also the special sections on " Fat " §§ 648 to 665.

738. Inclusions in Gametogenesis.

	1.	2.	3.	4.	5.	6.
Method.	Aoyama or Da Fano.	Kopschseries.	Chrome-osmium and iron hæmatoxylin or (Altmann).	Bouin and corrosive acetic and Ehrlich's hæm.	Champy-Kull.	Benda.
Mitochondria.	Either do not show or greyish or golden brown according as to whether sections have been toned.	Often will not show, or faintly yellowish, more rarely black or brown, but can often be decolorised rapidly in turps.	Black (or red).	Do not show.	Red.	Violet.
Golgi apparatus.	Black.	Black, and resists decolorisation in turps longer than mitochondria fat or yolk.	Rarely shows, when it does, black (or red), or not stained.	Does not show.	Rarely shows, if so, red.	Rarely shows, if so, violet.

	1.	2.	3.	4.	5.	6.
Method.	Aoyama or Da Fano.	Kopschseries.	Chrome-osmium and iron hæmatoxylin or (Altmann).	Bouin and corrosive acetic and Ehrlich's hæm.	Champy-Kull.	Benda.
Yolk granules.	Either will not show, greyish or golden brown, according as to toning.	Yellowish, or black, easily decolorised in turps.	May or may not go black (or unstained), very rarely red.	Not stained or yellowish.	Yellowish or black.	Yellowish or black.
Fat vacuoles.	Do not show.	Black, easily decolorised in turps.	Black in unstained preparation.	Not stained, washed away.	Black.	Black.
Chromatin granules.	Do not show, but may subsequently be stained in a basic colour, like methyl green or safranin.	Yellowish, will subsequently stain in hæmatoxylin or safranin.	Black to grey (or reddish purple).	Bluish to purple.	Blue.	Brownish yellow.
Nucleoli	Yellowish may take subsequent stain.	Yellowish.	Black or dark grey.	Reddish or reddish purple.	Red.	Violet or brown.

CHAPTER XXXI

VITAL STAINING *

PART I. GENERAL AND THEORETICAL

739. Types of Staining. Vital staining is the technique of colouring living cells. This may be brought about by the staining of preformed structures, by a general diffuse staining, or by the deposition of dyestuff as new intracellular formations (segregation, or "Speicherung"). Recent work has indicated that the type of staining is dependent to a certain extent upon the state of functional activity of the cells.

740. Vital Stains. Almost all the dyes employed for vital staining belong to one or other of the classes of acid or basic dyes. Acid dyes may be considered as salts of colour acids with bases, usually Na, K, or Ca; and basic dyes as salts of colour bases with acid radicles, usually —Cl or —SO_4. The following are the dyestuffs most frequently employed in vital staining :—

Acid Dyes. Trypan blue, trypan red, vital new red, isamine blue (pyrrol blue).

Basic Dyes. Neutral red, Janus green, brilliant cresyl blue, methylene blue and Bismarck brown.

Most acid dyes are water soluble, but insoluble in lipoid solvents. The majority of basic dyes, while soluble in water, have a certain degree of lipoid solubility.

741. General Methods of Staining. † Small aquatic animals can be stained by the addition of dyestuffs to the water in which they are kept. Terrestrial animals are injected with dilute solutions, the more diffusible dyes being injected subcutaneously or intraperitoneally, and the less diffusible intravenously. These methods are usually described as *intra vitam* staining, or staining *in vivo*. Staining of teased tissues, or isolated cells in dilute dye solutions, is known as *supra vital* staining, and is the method usually employed with basic dyes. One of the best means of studying vital staining is to employ tissue cultures—vital staining *in vitro*. Special irrigation apparatus for bathing cells with dye solutions has been devised by some workers (see Nagel, *Zeit. f. Zellforsch.*, ix, 1929, p. 346).

742. Vital Staining by Segregation. The acid dyes employed for vital staining, on entering living cells, are segregated as

† For Simpson's coverslip method, see § 872.

* By R. J. L.

granules or vacuoles in their cytoplasm. Thus there is brought about " an actual accumulation within the cell of vital dye-stuffs in fluid, high-colloidal, flocculate or crystalline form " (EVANS and SCOTT, *Contrib. to Embry.*, x, 1921, p. 1). There appears to be a protein substance associated with the dye droplets, at least in advanced stages of segregation (CHLOPIN, *Zeit. f. Zellforsch.*, xi, 1930, p. 316). According to SCHULEMANN (*Biochem. Zeit.*, lxxx, 1917, p. 1) the rate of diffusion of dyestuffs is the factor which determines their suitability as vital dyes. Highly diffusible dyes spread rapidly through the animal body, and are rapidly excreted. Indiffusible dyes and colloids when injected subcutaneously are deposited at the site of inoculation. The best dyestuffs for vital staining are those with medium rates of diffusion. Both SCHULEMANN (*Biochem. Zeit.*, lxxx, 1917, p. 1) and VON MÖLLENDORFF (*Ergeb. Physiol.*, xviii, 1920, p. 141) have concluded that chemical constitution has no direct relation to vital staining properties, but CAPPELL (*J. Path. and Bact.*, xxxii, 1929, p. 595) and WALLBACH (*Arch. f. expt. Zellforsch.*, x, 1931, p. 383) do not agree with this conclusion.

In addition to acid dyes, " vital staining " by segregation can be brought about by other means, *e.g.* with colloidal metals (collargol), india ink, and certain iron compounds.

The segregation of acid dyes is a relatively slow process compared with the staining of cell vacuoles by basic dyes. Furthermore, not all cells segregate acid dyes. Amongst those which do are macrophages, fibroblasts, fat cells of the connective tissues, Küpffer cells and parenchyma cells of the liver, capillary epithelium of the bone marrow and adrenal, reticulum cells of the lymph nodes, thymus and spleen, and kidney epithelium cells.

For further information concerning the cells which stain *intra-vitam* with acid dyes see VON MÖLLENDORFF (*Abderhalden's Hdb. biol. Arbeitsmeth.*, Sect. V., Part 2, 1921, p. 97), GLASUNOW (*Zeit. f. Zellforsch*, vi, 1928, p. 773), CAPPELL (*J. Path. and Bact.*, xxxii, 1929, p. 595), and LUDFORD (*Proc. R.S.B.*, civ, 1929, p. 493, and *ibid.*, cviii, 1931, p. 270).

743. Staining of Cytoplasmic Bodies with Acid Dyes. Acid dyestuffs rarely stain cytoplasmic inclusions, but instances have been recorded : these include protein granules in epithelial cells of the intestine of young mice, the secretion in mammary gland cells, and in certain of the ductless glands (VON MÖLLENDORFF *loc. cit.*, 1921) and granules (keratohyalin) in epidermal cells growing *in vitro* (LUDFORD, *10th Sci. Rep. Imp. Cancer Res. Fd.*, 1932, p. 169). Staining of preformed inclusions in invertebrate cells has been described by CHLOPIN (*Arch. expt. Zellforsch.*, iv, 1927, p. 462).

744. Staining of Preformed Structures with Basic Dyes. According to VON MÖLLENDORFF (*Abderhalden's Hdb. biol. Arbeitsmeth.*, Sect. V, Part 2, 1921, p. 97) the best basic dyes for staining cell

vacuoles and granules are those which, while water soluble, are only slightly soluble in lipoids, but are readily flocculated in contact with acid colloids, *e.g.* neutral red. SEKI (*Zeit. f. Zellforsch.*, xix, 1933, p. 289) also concludes that an intense granular staining depends essentially upon a reciprocal precipitation between acid substances in the watery phase of the cell granules, and the basic dyestuff. Probably because of their lipoid solubility, basic dyes such as neutral red, and brilliant cresyl blue readily penetrate all types of cells and quickly stain cytoplasmic vacuoles and granules. Also they stain yolk granules in embryonic tissues and dye droplets formed as the result of vital staining with acid dyes (VON MÖLLENDORFF, *loc. cit.*, 1921). Gatenby reports that in germ cells neutral red stains the γ-granules (rubrophile granules) in insects, annelids and mammals, and the proacrosome in many insects. In human spermatocytes needle-shaped crystals are also stained (GATENBY, *Anat. Rec.*, xlviii, 1931, p. 121).

745. Segregation of Basic Dyes. Besides staining preformed structures, basic dyes may under certain conditions give rise to new cytoplasmic formations. CHLOPIN (*Arch. expt. Zellforsch.*, iv, 1927, p. 462) has shown that with intense staining, newly formed dye droplets contain varying amounts of a substance, which he regards as protein from the way in which it is retained after fixation. This substance he calls the "Krinom." In fixed tissues it can be stained with basic anilin dyes.

LUDFORD (*Proc. R.S.B.*, cviii, 1931, p. 270) demonstrated that both acid and basic dyes when injected into the living animal are segregated in the liver cells in the same manner, in the same region of the cells, next to the intercellular bile canaliculi. He has therefore concluded that a basic dye such as neutral red on entering a cell is flocculated at the surface of any droplets and granules that may be present, possibly only if they are of a more acid character. Excess of the dye then becomes deposited as new cytoplasmic formations (*Biol. Rev.*, viii, 1933, p. 357). When working with basic dyes it is essential to bear in mind the possibility that coloured droplets which appear in cells may have been induced by the presence of the dyestuff, and not have been preformed. Failure to recognise this possibility has led to faulty conceptions of cytoplasmic structure.

746. Diffuse Staining of Cells. Certain basic dyes stain living cells diffusely. According to VON MÖLLENDORFF (*loc. cit.*, 1920) these are readily soluble in lipins, but show no disposition to be flocculated by acid colloids. Some have been found neither to stain preformed structures, nor to be segregated (LUDFORD, *loc. cit.*, 1931). Others, which are flocculated to a slight extent, stain cell granules faintly, and at the same time stain the cytoplasm diffusely. The following dyes have been employed for vital staining :—

Diffuse Staining. Rhodamine B, safranin G and diamant-fuchsin.

Diffuse Staining with Faint Granule Staining. Crystal violet 6 B, methyl violet 5B, auramine and malachite green.

Dead cells stain diffusely with most dyestuffs. Intra-vitam staining with trypan blue can therefore be employed for detecting centres of necrosis in the animal body. Dyestuffs which have been segregated by living cells become spread diffusely after cell death except when appropriate fixation has been employed.

According to Nassonov (*Zeit. f. Zellforsch.*, xi., 1930, p. 177 ; *Protoplasma*, xv., 1932, p. 239), Wallbach (*Zeit. f. Zellforsch.*, xiii., 1931, p. 180) and Alexandrov (*Protoplasma*, xvii., 1932, p. 161), dyestuffs which are segregated under normal conditions of functional activity, fail to be segregated under certain abnormal metabolic conditions (anaerobiosis and acidosis), and instead stain the nucleus and colour the cytoplasm diffusely.

747. General Technical Procedure. (1) Sterilise dye solutions before injecting by heating for not less than ten minutes in a test-tube contained in a beaker of boiling water. Heat-labile dyestuffs should be passed through a Berkefeld filter. (2) Swab the skin with alcohol where the injection is to be made. (3) Massage the site of subcutaneous injections so as to spread the dye. (4) After staining, tissues can be teased out in Ringer solution, and the cells examined in the living condition. Small pieces of connective tissue should be spread out rapidly on a dry slide and a drop of Ringer solution added immediately, and covered with a coverslip.

PART II. VITAL STAINING WITH ACID DYES AND RELATED SUBSTANCES

748. Trypan Blue. This is one of the best acid dyes for *intra-vitam* staining, and can be well retained in fixed tissues.

Preparation. Dissolve 0·5 grm. in 100 c.c. of distilled water, saline, or Ringer solution—some workers use a 1 per cent. solution. A solution in distilled water is satisfactory for most purposes. Filter. Heat at temperature of boiling water for ten minutes to sterilise. Old solutions should not be used as alterations in colloidal state occur after a time, and the dye becomes more toxic. It is undesirable to use solutions more than a month old. The dye solution is best kept in an ice safe.

Methods of Staining and Amounts to Inject. Inject either subcutaneously, intraperitoneally or intravenously. The following amounts are recommended for subcutaneous injections :—

Mice (per 20 grm. bodyweight) . .	0·5 to 1 c.c.
Rabbits (per 1,000 grm. bodyweight) .	10 to 15 c.c.
Frogs (per 20 grm. bodyweight) . .	0·5 to 1 c.c.

Inject at intervals of three to five days.

Staining occurs after the first injection, and increases in intensity with repeated injections. Fix twenty-four hours after the last injection.

Fixation and Staining. (*a*) *Frozen Sections.* Fix small pieces of tissue in 40 per cent. formalin for twenty minutes to two hours according to size; or in 10 per cent. formalin in normal saline overnight. Cut sections with a CO_2 freezing microtome. Neutral red, or carmine are good for post-vital staining. Make up neutral red as follows—dissolve 1·0 grm. of neutral red in 1,000 c.c. of distilled water, and then add 2 c.c. of a 1 per cent. solution of glacial acetic acid. Stain sections rapidly; less than a minute is usually sufficient. Rinse with distilled water, and blow off excess water from slide. Bring direct to absolute alcohol, and after clearing in xylol, mount in Canada balsam. For details of frozen section technique see §§ 223-232.

In many tissues mitochondria can be demonstrated as well as dye droplets by staining sections with Hollande's iron carmine (see § 259). Ludford has employed this method in the study of *intra-vitam* staining of liver and kidney cells (*Proc. R.S.B.*, ciii, 1928, p. 288). Stain sections for a minute or less in the carmine solution. Blacken them in iron alum, and if necessary leave in this solution till they are sufficiently differentiated. Wash in running water. Dip in pyridine, and wash again in running water. Dehydrate, and mount in Canada balsam. In successful preparations the mitochondria are dark brown, and the trypan blue droplets bluish-black to pale blue.

(*b*) *Paraffin Sections.* Trypan blue can be retained in paraffin sections after fixation by various methods. Thus tissues can be fixed in 5 or 10 per cent. formalin, concentrated sublimate solution, or in mixtures of these two (8·5 parts of saturated corrosive sublimate to 1 part of 40 per cent. formalin). The potassium bichromate technique of Altmann for demonstrating mitochondria has also been successfully employed. PFUHL (*Zeit. f. Zellforsch.*, xiii, 1931, p. 783), who investigated the action of a large number of fixatives on tissues vitally stained with this dye, came to the conclusion that Heidenhain's "Susa" gave the best results. This fixative is best made up fresh, as described in § 93.

The amount of sublimate precipitated in the tissues is very slight, so that it is usually unnecessary to employ any methods for removing it. The treatment of vitally stained tissues, or of sections of such tissues, with iodine dissolved in alcohol, for the purpose of removing sublimate should be avoided as the vital staining is seriously impaired.

After fixation, which should vary from one to twenty-four hours, according to the size of the pieces, they are transferred direct to 80 or 90 per cent. alcohol which should be changed several times.

(*c*) *Staining of Sections.* The various carmine stains are specially

suitable for staining sections. LUDFORD (10*th Sci. Rep. Imp. Cancer Res. Fd.*, 1932, p. 169) recommends Rawitz's carm-alum, made up as described in § 254. Progressive staining is desirable so as to avoid the necessity for using acid fluids in order to differentiate.

749. Golgi Apparatus and Trypan Blue Staining. JASSWOIN (*Zeit. f. Zellforsch.*, ii, 1925, p. 741), NASSONOW (*ibid.*, iii, 1926, p. 472), MAKAROV (*Arch. Russes d'Anat., d'Hist. et d'Embryol.*, v, 1926, p. 157) and GLASUNOW (*Zeit. f. Zellforsch.*, vi, 1928, p. 773) have described a topographical relationship between the Golgi apparatus and dye droplets in a variety of cells. LUDFORD (*loc. cit.*, 1928) was able to demonstrate the Golgi apparatus in vitally stained kidney cells by the modified Mann-Kopsch method. Small pieces of the vitally stained kidney were cut up with a sharp knife and fixed overnight in corrosive-osmic solution. Then washed for one hour in repeated changes of distilled water. The material was then transferred to 2 per cent. osmic acid, and kept in an incubator at 35° C. for two-and-a-half days: repeatedly washed with warm water, and incubated a further day in distilled water.

This method gave good results with kidney cells, but was not so satisfactory with liver cells. However, it was found possible to demonstrate trypan blue droplets in the latter by counterstaining sections with neutral red.

750. Vital Staining with Trypan Blue in vitro. Tissue cultures can be vitally stained either by adding the dyestuff to the medium employed for explantation, or by adding the dye in solution to cultures after growth is well advanced. The dye is usually applied in a 0·5 per cent. solution in Ringer solution, or distilled water. It should be applied by means of a finely pointed pipette. To each coverslip culture add a small drop, just sufficient, on diffusion, to tint the medium a pale blue colour. Before fixation, wash the cultures with Ringer solution. Fixation in "Susa" for ten to twenty minutes followed by staining in Rawitz carmalum (see § 254) for two to four minutes usually gives good preparations.

For routine method of fixing and staining vitally stained cultures, see DUNN (*Arch. f. Zellforsch.*, xvi, 1934, p. 361).

751. Vital Staining with Pyrrol Blue (Isamine Blue). This dyestuff was largely used by Goldmann, but has been superseded by trypan blue, which he also introduced as a vital stain. It is deposited at the site of subcutaneous injections, and then goes slowly into solution again giving rise to a general vital coloration. For vitally staining mice, 0·5 to 1·0 c.c. of a 0·5 or 1·0 per cent. solution of the dye should be employed. It is difficult to retain in fixed preparations.

CAPPELL (*J. Path. Bact.*, xxxii, 1929, p. 595) recommends Zenker's fluid as the best fixative, but dehydration must be rapid, and the

weaker alcohols avoided as much as possible. Bouin's fluid can be used for fixing spread tissues and for cellular exudates.

752. Staining with Vital New Red. This dye is a good vital stain, and is less toxic than trypan blue, but it has the disadvantage of being much more difficult to retain in fixed preparations. It is a convenient dye to employ in double-staining experiments with trypan blue, when only examination of the living tissues is intended.

Methods of Staining and Amounts to Inject. Inject either subcutaneously, intraperitoneally or intravenously. Mice (per 20 grm. bodyweight): 0·5 to 1·0 c.c. of a 1 per cent. solution daily. Rabbits (per 1,000 grm. bodyweight): 5 c.c. of a 5 per cent. solution. Guinea-pigs (per 100 grm. bodyweight): 2 c.c. of a 2·5 per cent. solution.

Fixation. The dye dissolves in the lower alcohols, but not in absolute alcohol, which may therefore be employed as a fixative. CAPPELL (*loc. cit.*, 1929) recommends corrosive sublimate solution, and states that Zenker or Bouin also are satisfactory with some tissues.

753. Staining with Diaminefast Scarlet. This is another suitable dyestuff to employ for double-staining experiments with trypan blue. It has the advantage of being retained well in fixed preparations. For mice (per 20 grm. bodyweight) inject 0·5 to 1·0 c.c. of a 1 per cent. solution, either subcutaneously, or intraperitoneally, and for rabbits (per 1,000 grm. bodyweight) inject 20 c.c. of a 5 per cent. solution.

According to CAPPELL (*loc. cit.*, 1929) the best fixatives are Bouin and corrosive-sublimate.

754. Staining with Carmine. Lithium carmine has the advantage of being well retained after fixation, and resists solution by the alcohols used for dehydration. It is, however, rather more poisonous than trypan blue. The best method to administer it is by intravenous injections.

(i.) **Preparation.** (1) Dissolve 2·5 grm. of carmine in 100 c.c. of a saturated solution of lithium carbonate. (2) Boil ten to fifteen minutes on a water-bath. (3) Filter immediately before using—filtration through a Berkefeld filter will ensure sterility.

(ii.) **Intravenous Injections.** With mice, injections should be made into the tail vein. It is advisable to dilute the stock solution to 20 per cent., and to commence by injecting 0·2 c.c. of this.

Repeated injections are necessary, and should be made daily if the condition of the animals will allow. The amount of the dye injected may be increased with later injections. A general vital staining is attained after five to eight days.

Rabbits may be given 10 c.c. of a 10 per cent. solution daily.

(iii.) **Fixation.** Tissues can be fixed in 10 per cent. formalin in

normal saline, and frozen sections cut and stained with Harris's hæmatoxylin. By careful staining with Hollande's iron-carmine method (see § 259) it is possible to demonstrate mitochondria together with the carmine granules.

For paraffin sections tissues are usually fixed in 85 to 95 per cent. alcohol, 10 per cent. formalin, or in corrosive sublimate. Formal-sublimate (8·5 parts of saturated sublimate to 1 part of 40 per cent. formalin) has also been employed. Iodine for the removal of sublimate from sections must be employed with extreme care, or decoloration will ensue. Suitable stains for sections are Mayer's hæm-alum, methylen blue or Pappenheim's methyl green and pyronin. Chrome-osmic methods (Altmann and Schultze) for the demonstration of mitochondria have been successfully employed with tissues vitally stained with carmine.

755. Vital Staining with Carmine in vitro. Carmine is frequently employed for vitally staining tissue cultures. Small drops of a 20 per cent. solution of the stock solution should be applied to cultures in the same manner as with trypan blue. They can be fixed in 10 per cent. formalin in saline, and stained with Harris's hæmatoxylin or Mayer's hæm-alum.

756. Other Acid Dyes Suitable for Vital Staining. Other acid dyes stated by von Möllendorff to be suitable for *intra-vitam* staining are trypan red, dianil blue, cinnebar scarlet G, brilliant congo G, Hessisch brilliant purple, vital new orange.

757. Staining with India Ink. This is particularly suitable for demonstrating the phagocytic properties of cells. It should be injected intravenously.

(i.) **Preparation.** (1) Dilute the India ink (Higgins' is usually employed) 10 to 50 per cent. with distilled water.

(2) Filter through two thicknesses of ordinary filter paper to remove the coarser particles.

(3) Boil ten minutes to sterilise.

(ii.) **Intravenous Injections.** Cappell (*loc. cit.*, 1929) recommends for mice (20 grm. per bodyweight) 0·4 c.c. of a 10 per cent. dilution, and for rabbits (1,000 grm. per bodyweight) 2 c.c. of a 50 per cent. solution.

(iii.) **Fixation.** Since the ink particles are insoluble, any good fixative can be used. In double staining with trypan blue, "Susa" fixation, followed by staining with Rawitz carmine, is recommended.

(iv.) **For Tissue Cultures.** The addition of dilute India ink to tissue cultures is a convenient method for investigating the phagocytic properties of cells *in vitro*. When used with trypan blue it affords a means of distinguishing between phagocytosis and segregation (Ludford, *loc. cit.*, 1933).

758. Iron Compounds as Vital Stains. Iron compounds for cytological studies have been employed amongst others by

Okkels (*Arch. f. exper. Zellforsch.*, viii, 1929, p. 432), Cappell (*loc. cit.*, 1929), Chlopin (*Zeit. f. Zellforsch.*, xi, 1930, p. 316), and Makarov (*ibid.*, xix, 1933, p. 28). Saccharated oxide of iron is regarded by Cappell as the most useful of all the suspensoids for vital staining. He recommends a 10 per cent. solution by weight in distilled water. This should be boiled to sterilise.

Intravenous Injections. For mice, 0·3 c.c. of a 10 per cent. solution to start with, and with later injections the amount can be increased according to the tolerance of the animals. For rabbits, 5 c.c. of a 25 per cent. solution by weight (Cappell, *loc. cit.*, 1929).

Fixation. Twenty per cent. formalin, or absolute alcohol. Some workers employ a mixture of these two reagents.

Demonstration of the Iron in Sections. Either the Prussian blue reaction with potassium ferrocyanide and hydrochloric acid, or the Turnbull blue method with ammonium sulphide followed by acidulated potassium ferricyanide (see § 674). Sections can then be stained with alum-carmine or para-carmine.

PART III. VITAL STAINING WITH BASIC DYES

759. Vital Staining of the Nucleus. There is a general consensus of opinion that the nucelus does not stain under normal conditions of metabolic activity, but some basic dyes, such as methylen blue, stain nucleoli faintly, without killing the cells. According to Russell (*J. Expt. Med.*, xx, 1914, p. 545) gentian violet stains the nucleus and chromosomes of embryonic and adult tissues of the frog, in tissue cultures. He employed this dye in dilutions of 1 in 4,000 to 1 in 20,000 added to the culture medium. Staining of the nucleus has been described under abnormal conditions of metabolism, *e.g.* under anaerobic conditions, and also in a state of acidosis (see § 746).

760. Vital Staining of Mitochondria. Janus green is most extensively employed for the vital staining of mitochondria. According to Cowdry (*Am. J. Anat.*, xix, 1916, p. 423) its action depends upon the presence in its molecular structure of the diethylsafranin group, and this alone is capable of staining mitochondria. Other dyes containing the same group, and possessing the same properties, are Janus blue, Janus black and Janus grey. A good preparation of the dye is essential for the best results. That of Höchster Farbewerke is generally recommended. There is conflict of opinion as to whether mitochondria can be stained with these dyes without cellular injury, but Cowdry (*Inter. Monatschr. Anat. Phys.*, xxxi, 1914, p. 267) observed neutrophile leucocytes with vitally stained mitochondria performing amœboid movements, and phagocytosing foreign particles. The Janus dyes are employed in concentrations of 1 in 10,000 to 1 in 500,000 in physiological saline. The latter

dilution according to Cowdry (*Amer. Nat.*, lx, 1926, p. 157) will stain mitochondria of human lymphocytes selectively.

761. Methods for Staining Mitochondria with Janus Green. Supravital. Tease out small fragments of tissue in saline in a small Petri dish. Draw off saline with pipette, and replace with a shallow layer of the dye solution ($\frac{1}{20000}$ to $\frac{1}{50000}$). Replace the lid of the dish. With tissues of homoiothermal animals place in incubator at body temperature for ten to twenty minutes, and then mount for examination in saline.

Supravital staining on prepared slides is especially recommended for isolated cells, such as those of the blood and bone marrow.

SCOTT'S method (*Anat. Rec.*, xxxvii, 1928, p. 233) is carried out as follows :

Preparation of the Slides.—(i.) Prepare a 1 per cent. solution of the dye in absolute alcohol, and filter after an hour to remove any undissolved dye. (ii.) Employing a pipette with a bore of approximately 1 mm., allow three drops of the dye solution to fall on one end of each of several cleaned slides. (iii.) Smear along the length of each slide with the edge of another slide, as if making blood smears. The alcohol evaporates, leaving a fine, even film of the dyestuff.

Method of Staining.—(i.) Warm the prepared slides and some cover-slips in the incubator. (ii.) Apply small drops of blood, or suspension of cells, in saline to prepared slides and cover immediately with the warmed cover-glasses. Press gently to spread the drops and seal the edges of the cover-glasses with melted vaseline. Cells of compact tissues can be stained by cutting the latter with a sharp knife, and pressing the cut surface gently on to the prepared slide, then adding a small drop of saline, and applying a cover-glass. (iii.) Examine if possible in a warm chamber (37° C.) or on a warm stage, otherwise it is usually necessary to place the slides in an incubator for a few minutes.

Fixation of the Vital Staining. (i.) Wipe vaseline from the edge of the cover-glass. (ii.) Remove cover-glass by sliding it towards the nearest edge of the slide. This helps to spread the cells evenly and permits of permanent preparations being made from both cover-glass and slide. (iii.) Dry cover-glass and slide quickly in the air. Rapid drying is essential to avoid distortion of the cells. (iv.) Complete drying, if necessary, in a vacuum desiccator for two to four hours, or by shaking in several changes of anhydrous ether. Treatment with ether is particularly recommended with bone marrow, in order to remove fat as well as moisture. (v.) Clear in xylol, and mount in balsam.

Intra-vitam Staining. Inject solutions of the dye in dilutions of 1 in 20,000 to 1 in 50,000. After ten to thirty minutes kill the animal, and tease out the tissues in saline. Some authors recommend exposing the tissues to the air for a minute or two before laying on the coverslip.

Staining by Perfusion. Kill the animal and inject the dye solution in a dilution of 1 in 15,000 in isotonic salt solution, through the aorta. Then remove tissues and transfer to saline at room temperature (Bensley, *Am. J. Anat.*, xiii, 1911–12, p. 297).

762. Staining with Janus Green in vitro. Tissue Cultures. With cover-glass cultures, raise the coverslip, and fill the well of the hollow slide with the dye solution. Strangways (Cambridge, 1924) recommended 1 in 40,000 dilutions. The culture can be transferred to the incubator for five to ten minutes, but is best examined under the microscope on a warm stage, or in a box maintained at body temperature.

763. Fixation of Vitally Stained Mitochondria. No method has yet been devised which is generally applicable to cells vitally stained with Janus green, or the other Janus dyes. Temporary fixation of the dye is possible in isolated cells, or in cells growing in tissue cultures, by Lewis method (*Amer. J. Anat.*, xxx, 1922, p. 39). With tissue cultures Lewis advises raising the coverslip and placing a small flake of iodine at the bottom of the well of the hollow slide. With isolated cells teased out on a slide. A few flakes of iodine are heated in a small test-tube, and the iodine vapour allowed to come in contact with them by tilting the test-tube. By this method the vitally stained mitochondria lose their colour, becoming dark brown in appearance, but are well preserved temporarily.

764. Staining of Mitochondria with Other Basic Dyes. Ludford (*Arch. f. exper. Zellforsch.*, xvii, 1935, p. 339) has pointed out that mitochondria can be well stained with methylene blue, especially in cells of tissue cultures. This staining can also be temporarily fixed by the iodine vapour method of Lewis (see above). For vitally staining cultures proceed as described above for staining with Janus green, employing methylene blue in a dilution of 1 in 10,000 in Ringer solution for four to eight minutes. For *intra-vitam* staining, inject a 0·5 per cent. solution in Ringer solution, and kill animals after fifteen to thirty minutes. For supravital staining, incubate teased tissues in a 1 in 10,000 dilution in Ringer solution, for fifteen to thirty minutes.

Mitochondria vitally stained with this dye are rapidly decolourised on exposure to bright illumination, which also inhibits the staining process.

Other basic dyes which have been found to stain mitochondria of some cells are toluidine blue and brilliant cresyl blue.

765. Vital Staining and the Golgi Apparatus.* No dyestuff has yet been discovered to stain the Golgi apparatus specifically. Most investigators have not found it to stain with the dyestuffs usually employed for vital staining. However, staining of the Golgi bodies in invertebrate germ cells has been reported. This literature was reviewed by Bowen (*Anat. Rec.*, xxxviii, 1928, p. 293). In fibroblasts growing in tissue cultures Ludford (*loc. cit.*, 1935)

* Neutral red has been claimed to stain the dictyosomes by Perrincito, Grabowska, Tuzet, and Wilson in snail, crayfish and scorpion. These appear to be the only cases in the literature (see *J. Morph.*, 1937).—Eds.

found that by staining with methylene blue it was possible to demonstrate the Golgi apparatus as a colourless reticulum against the blue stained cytoplasm.

766. The Staining of Cytoplasmic Granules and Vacuoles. Neutral red is one of the best dyes for this purpose. Aquatic animals, *e.g.* tadpoles, can be stained by keeping them in weak solutions—1 in 200,000, or less. With stronger concentrations staining is more rapid, but segregation of the dye may occur.

The various methods of staining with Janus green (see § 761) are also applicable to neutral red. Neutral red is frequently used together with Janus green. Mitochondria are then stained with the latter, and vacuoles and granules with the former. A mixture of neutral red and methylen blue has been found to give similar results. A temporary fixation of the staining is possible with the iodine vapour method of Lewis.

Supravital. Use dilutions of 1 in 20,000 to 1 in 50,000, or less. For staining blood, bone marrow and isolated cells, prepare slides as described in § 761, employing neutral red in a 1 per cent. solution in absolute alcohol. For double staining, with Janus green, mix 1 per cent. alcoholic solutions of the dyes before applying to the slides.

Intra-vitam. For staining connective tissue cells, inject solutions of 1 in 50,000, or less, in saline, subcutaneously. Kill animals and remove tissues for examination after ten to twenty minutes.

In order to stain pancreas and liver cells of small laboratory animals stronger solutions are usually injected intraperitoneally. Small injections at intervals of thirty minutes, or 1 c.c. of a 0·5 per cent. solution have been employed.

In Vitro. Solutions of 1 in 50,000 to 1 in 100,000 in saline usually give the best results. For the coverslip method of staining blood and Protozoa, see under "Blood," § 872. This is Simpson's method.

767. Preservation of Neutral Red Staining. The preservation of neutral red staining presents considerable difficulties. Most of the methods which have been described have a limited range of application. LUDFORD (*Proc. R. S. B.*, cvii, 1930, p. 101) has employed Gardner's method for cytological work (*Proc. Soc. Expt. Biol. Med.*, xxiv, 1926, p. 646), making up the fixative as follows :—

43 c.c. of Zenker's fluid (without acetic acid).
 7 c.c. of formol to which 2 drops of normal NaOH have been added.

Gardener's Technique. (1) Fix for twenty-four hours. (2) Cut up tissues into small blocks, not more than 2 mm. in thickness, and transfer to fresh Zenker's fluid (without acetic acid). (3) Commence dehydration any time within the next two days by washing for fifteen minutes, then blot the small blocks of tissue. (4) Transfer to 80 per

cent. alcohol for ten minutes. (5) Pass through graded mixtures of 95 per cent. alcohol and benzene in the following proportions: 9 : 1, 8 : 2, 7 : 3, 5 : 5, 3 : 7, 2 : 8, and 1 : 9, leaving ten minutes in each. (6) Clear in benzene, one or more changes for an hour or two. (7) Imbed in benzene saturated with wax at 37° C. for an hour, followed by paraffin at 56° C. (four changes in thirty minutes). Sections can be mounted unstained or counterstained with Harris's hæmatoxylin.

Silver Impregnation Method of Yamasaki (*Arbeit. anat. Institut kaiserlich.-jap. Univ. Sendai*, xv, 1933, p. 7). According to Yamasaki neutral red droplets after fixation in alkaline-Müller-formalin are argentophile, while trypan blue droplets are not (*ibid.*, xv, 1933, p. 19). He demonstrates neutral red droplets by the following method :—

(1) Fix in Müller's fluid, 18 c.c.; formalin, 2 c.c.; 40 per cent. KOH, 8 drops—for twelve to twenty-four hours. (2) Wash with repeated changes of distilled water for twelve to twenty-four hours. (3) Transfer to 0·75 per cent. silver nitrate solution for twenty-four hours. (4) Wash in distilled water, then reduce for twenty-four hours in hydroquinone, 1·5 gm.; formol, 5 c.c.; distilled water, 100 c.c. (5) Wash rapidly and dehydrate in alcohols and imbed. Sections should be rapidly differentiated in a 5 per cent. watery solution of sodium thiosulphate. Then well washed and stained with alum carmine.

774. Hu's Technique (*Proc. Soc. Expt. Biol. Med.*, xxix, 1931, p. 258). Hu has taken advantage of the fact that neutral red is only slightly soluble in aqueous or alcoholic solutions containing corrosive sublimate, and devised the following technique. Four solutions are first prepared.

(A) The fixing solution. Dissolve a little neutral red in 15 c.c. of 40 per cent. formalin in a flat dish. Add 85 c.c. of Zenker's fluid. Stir with a glass rod and filter off the precipitated dye.

(B) Dehydrating solution. To 100 c.c. of absolute alcohol add 12 c.c. of the same reagent saturated with $HgCl_2$, then sufficient neutral red until some of it remains undissolved at the bottom of the bottle.

(C) Aqueous solution. To 100 c.c. of distilled water add 2 c.c. of a saturated aqueous solution of $HgCl_2$, and then saturate with neutral red. Filter.

(D) Counterstaining solution. Saturate some of solution (C) with methylen blue. Filter. This must be prepared fresh each time it is required.

Method of Procedure.—(1) Fix in solution (A) blocks of tissue about 1 c.m. square and 3–4 mm. thick for twenty-four hours. (2) Dehydrate in three changes of solution, (B) one or more hours each time, according to the size of the pieces. (3) Clear in two changes of xylol, three to four hours in each. (4) Imbed in paraffin and mount sections in the usual manner, after removing wax with xylol.

Counterstaining Sections.—(1) Remove wax in xylol. (2) Wash off xylol with solution (B). (3) Wash off alcohol rapidly with solution (C). (4) Stain in solution (D) for about thirty seconds. (5) Remove excess of stain with solution (C). (6) Dehydrate with solution (B). (7) Clear in xylol and mount.

768. Demonstration of New Formation in the Cytoplasm of Cells after Neutral Red Staining. The Krinom (see § 745). After intense staining with neutral red, a substance is formed which

Chlopin has called the Krinom. It can be demonstrated by fixation in Helly's fluid (Zenker-formol) and counterstaining with thionine or eosin-azure. With the latter staining it appears blue against the pink background of the cytoplasm.

LUDFORD (*Proc. R. S. B.*, cviii, 1931, p. 270) has demonstrated the same formations by fixation in Champy's fluid, bleaching section in hydrogen peroxide (1 part of H_2O_2 to 4 parts of 80 per cent. alcohol), and then counterstaining with acidulated neutral red (see § 748).

769. Other Basic Dyes for Staining Cytoplasmic Vacuoles and Granules. Other dyes which may be employed, and stain similarly to neutral red, are neutral violet, brilliant cresyl blue, Nile blue sulphate, Bismarck brown, Nile blue chlorhydrate, toluidine blue and thionine. They can be applied to cells in the same way as neutral red.

770. The Staining of Fat Droplets. Fat can be stained by feeding animals with food to which a fat soluble dye, such as Sudan III., or scarlet R, has been added. The dye may be dissolved in olive oil, and this mixed with dry food, or the dye can be well mixed with fatty foodstuffs. Solutions in alcohol and acetone have been injected directly into animals (see HADJIOLOFF and OUZOUNOFF, *Compt. rendus*, cxiii, 1933, p. 1501). LUDFORD (11*th Sci. Rep. Imp. Cancer Res. Fd.*, 1934, p. 169) has devised a method of vitally staining fat by employing "solutions" of Sudan III. and Sudan black in serum. The method of preparation is as follows : A small quantity of the dye is added to freshly-prepared serum contained in a small test-tube, provided with a tight-fitting rubber stopper in order to prevent loss of CO_2. The test-tube is vigorously shaken from time to time and kept in a warm place. Within twenty-four to forty-eight hours the serum becomes coloured. It is then centrifuged and the coloured transparent fluid pipetted off. This is relatively stable, and can be used for *intra-vitam* or supravital staining, or for staining cells in tissue cultures.

The basic dyestuff Nile blue which is soluble in water, as well as in lipoid solvents, has also found employment for the staining of fatty compounds. According to the recent work of LISON (*Bull. d'Hist.*, x, 1933, p. 237 ; *ibid.*, xii, 1935, p. 279) the blue staining with this dye is of no histochemical significance, but the rose coloration signifies the presence of an unsaturated glyceride (trioleine). It is not possible to distinguish between different classes of fatty compounds by vital staining.

PART IV. SPECIAL METHODS

771. Vital Staining with Leucobases. Roskin and his collaborators (ROSKIN and SEMENOFF, *Zeit. f. Zellforsch.*, xix, 1933, p. 150 ; ROSKIN and MASLOWA, *Zeit. f. wiss. Mikros.*, lii, 1935, p. 309),

have described a technique for vital staining with leucobases of certain basic dyes. Their method of preparation is as follows: To 100 c.c. of 0·01 per cent. dye solution are added 1 to 2·5 c.c. of N/10 sodium hyposulphite and 1 to 4 c.c. of N/10 HCl. The mixture is well stirred with a glass rod and kept at room temperature in the dark. According to the dyestuff and the concentration of the reagents employed, colourless leucobases are formed in from two to twenty-four hours. They resist oxidation by atmospheric oxygen, but are re-oxidised if the medium is made alkaline or exposed to daylight, especially to direct sunlight. Leucobases have been prepared in this way from methylen blue, azure 1, thionine, toluidine blue and brilliant cresyl blue. Mixtures of two leucobases can be employed, *e.g.* of thionine and azure 1. Staining is carried out by adding one or two drops of the leucobase to cells contained in a drop of physiological saline. The method is described as being suitable for differentiating between different types of cells and for the study of oxydation-reduction processes. Roskin and his co-workers have described the reaction of many different kinds of cells to these leucobases, *e.g.* mast cells of the frog when treated with a mixture of the leucobases of thionine and azure 1 acquire a light blue coloration of their nuclei, rose-coloured cytoplasm and an intense red coloration of their granules.

772. Indirect Vital Staining. Karczag and Paunz (*Deut. med. Woch.*, xlix, 1923, p. 1231) have introduced a technique which they term "indirect vital staining." Dyestuffs (fuchsin S, light green, water blue) are injected subcutaneously in 2·5 per cent. solutions in saline. Rabbits receive in the course of a day 5 grm. of fuchsin, 3 grm. of light green and 4 grm. of water blue. Tissues may be fixed in formalin and frozen sections cut, or fixed in a mixture of formalin and acetic acid and dehydrated and imbedded as soon as possible. The sections are faintly coloured or colourless, as the dyes are changed to colourless carbinol compounds. They are converted to the coloured forms by treating sections for several hours with 0·1 per cent. HCl.

773. Staining of Intercellular Structures. The matrix of growing bone can be stained by feeding young growing animals with madder or with alizarin. Gottlieb (*Anat. Anz.*, xlvi, 1914, p. 179) injected 1 per cent. solutions of sodium alizarin sulphonate in saline or Ringer solution. The free calcium salts of the new-forming bone are stained, but not the cells.

Some staining of the elastic fibres of connective tissue occurs with trypan blue. For staining amyloid, Congo red has been employed.

774. Vital Staining as a Method for Investigating the pH of Cells. Rous (*Journ. Expt. Med.*, xli, 1925, pp. 379, 451, 739) has employed litmus and the phthaleins for studying the *p*H of the tissues.

With litmus the vacuoles of macrophages stain red, thus indicating their acid character. NASSONOW (*Zeit. f. Zellforsch.*, xi, 1930, p. 177; *Protoplasma*, xv, 1932, p. 239) has devised a technique for studying intracellular *p*H using neutral red and other basic dyes. CHAMBERS (*Proc. Soc. Expt. Biol. Med.*, xxvii, 1930, p. 809) found that methyl red vitally stained cells when the *p*H of the surrounding medium was definitely below 6·0. For investigating intracellular hydrogen ion concentration the micro-injection technique is particularly useful. For references to papers by Chambers and his collaborators, see CHAMBERS and LUDFORD (*Proc. R. S. B.*, cx, 1932, p. 120).

775. Vital Staining as a Method for Investigating the Mechanism of Mitosis. Aberrations of mitosis are brought about by vital staining. According to VON MÖLLENDORFF (*Zeit. f. Zellforsch.*, xxiii, 1936, p. 746) neutral red in a concentration of 1 in 200,000 interferes with mitosis in tissue cultures. POLITZER (*Biochem. Zeit.*, cli, 1924, p. 43) found that a concentration of 1 in 150,000 resulted in a decrease in the number of mitoses in epidermal cells of salamander larvæ after two hours, and there were many "pseudo-amitoses." Nile blue had a similar action. Auramine and brilliant cresyl blue arrested mitosis at metaphase. LUDFORD (*Arch. f. expt. Zellforsch.*, xviii, 1936, p. 411) found that auramine had a similar action *in vitro*, preventing the formation of the mitotic spindle.

Vital staining with methylen blue affords a means of demonstrating cells in division in tissue cultures, since dividing cells do not stain so intensely as "resting" cells (LUDFORD, *Arch. f. expt. Zellforsch.*, xvii, 1935, p. 339).

PART V. LITERATURE

776. General Reviews

VON MÖLLENDORFF, W., *Ergeb. Physiol.*, xviii, 1920, p. 141. CAPPELL, D. F., *J. Path. Bact.*, xxxii, 1929, p. 595. GICKLHORN, J., *Ergeb. Bio.*, vii, 1931, p. 549. NAGEL, A., *Oppenheimer's Hdb. Bioch. d. Mensch. u. d. Tiere*, i, 1933, p. 783.

777. Vital Staining for Specialised Researches

Protozoology

VONWILLER, P., *Abderhalden's Hdbuch. d. biol. Arbeitsmeth.*, Abt. V Teil. 2, 1921, p. 87.

Embryology

WINTREBERT, P., *Bull. Assoc. d. Anat.*, xxvii, Reunion Report, 1932.

Cellular Physiology and Pathology

LUDFORD, R. J., *Biol. Rev.*, viii, 1933, p. 357.

Cancer Research

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CHAPTER XXXII

THE TECHNIQUES FOR THE CULTIVATION OF VERTEBRATE TISSUES IN VITRO *

778. THE technique of tissue culture provides a means whereby the growth or the survival of tissues and cells may be observed in an environment apart from that of the intact organism. The behaviour of cell-types in mixed cultures and in pure strains in response to changes in the medium and to external stimuli, the interaction between cultures of different types of tissues growing side by side in the same medium, and the potentialities of embryonic rudiments and organs isolated from the influences of the rest of the embryo, have all been extensively studied by this technique of explantation. In 1907 Ross Harrison first demonstrated the growth of nerve fibres in fragments of nervous tissues explanted from frog embryos in adult frog lymph. More recently the technique has been modified in the direction of standardising the conditions favourable to the active growth of cultures over extended periods of time. Much has still to be accomplished in the control of the chemical composition of the media and of the gaseous environment of tissue cultures. It is no longer a phenomenon to obtain some sort of growth from any embryonic or fœtal tissue, and as FELL (*Brit. Journ. Radiol.*, viii, 1935, p. 27) has emphasised, " it is a waste of time and money to take up tissue culture merely for the sake of growing cells."

When sufficient technique has been acquired for the successful cultivation of simple embryonic tissues such as heart muscle (fibroblasts), iris epithelium, intestinal epithelium, etc., over a period of a month or more, attention should be given to the problem of controlling the composition of the media, and the gases in contact with it, as accurately as possible. The operation of cutting out a fragment of living tissue and placing it in a nutrient medium includes a wide range of possible errors and variable results. To eliminate some of these variations in experimental work, a large number of control cultures is desirable. The worker should also be familiar with the appearance of degenerating cells *in vitro*. Slight degrees of abnormality are often indicated only by changes in the form of the mitochondria and other cytoplasmic inclusions. At present the technique is largely dependent upon personal skill and the quality of cultures is apt to vary considerably in different laboratories engaged in tissue culture.

* By K. C. R.

It is not possible within the scope of a single chapter to describe more than the essential features of the technique and to emphasise the more common errors and pitfalls. A list of text-books for further study is recommended at the end of the chapter.

779. Preparation of Glassware and Instruments. All glassware coming in contact with the media and the tissues should be of Pyrex or Jena, non-alkali glass. Cleaning, prior to sterilisation, requires special attention. First boil in water with a refined soap, or if the glass is new, in 5 per cent. H_2SO_4. The remains of clotted plasma in pipettes or on coverslips may be removed in a boiling bichromate-sulphuric acid mixture such as:—

Pot. bichromate	4–6 grm.
Conc. H_2SO_4	6 c.c.
Water	100 c.c.

But great care must be taken to remove the acid subsequently by ammonia and then by prolonged washing. Wash finally in several changes of distilled water (free from heavy metals) and drain. Immersion in re-distilled 95 per cent. alcohol may be preferred before drying. Coverslips should be washed individually with great care and stored in absolute alcohol. After polishing with linen or silk, examine for traces of fluff and remove it by flaming, then store the coverslips in a Petri dish on a piece of greaseproof paper. Wrap the glassware in thin smooth paper before sterilising. Pipettes may be stored in large boiling tubes fitted with corks. It is convenient to have glassware sufficient for several weeks' work sterilised at the same time and stored in a clean cupboard.

Rubber stoppers and teats, when new, often require boiling in dilute alkali to remove traces of acid. Always sterilise rubber by placing it in a glass vessel in a little water in the autoclave.

Glassware to contain blood, freshly prepared plasma (up to forty-eight hours old) and saline solutions must be coated internally with refined paraffin wax. Fill the sterile vessels or tubes with melted wax which has been sterilised by heating. While they are still quite hot, spread a sterile towel on a table, empty each vessel of its wax and rest its mouth downwards on the towel to drain. Place a sterile cork or rubber stopper in each after the wax has set evenly on the interior. Cover the corks and stoppers with sterile paper caps and a rubber band if they are to be stored for any length of time.

780. Saline Solutions. The popular conception that living tissue can be placed without harm in any sterile physiological salt solution containing the approximate proportions of salts obtaining in plasma is quite erroneous. The saline solutions which come in contact with the living tissues to be explanted and which form part of the media, must be prepared with extreme care and accuracy. A short period in an inaccurately prepared saline of unsuitable *p*H is sufficient to hinder or entirely suppress the

growth of a tissue in nutrient media. All glass containers should be of hard glass, paraffin coated and plugged with rubber or paraffined corks to prevent gaseous exchange. Only purified analytical reagents should be used. The solutions are made up in double distilled water and the salts added separately, in strict order, allowing each to dissolve before the next is added.

Tyrode solution (for warm-blooded animals). (TYRODE, *Arch. Internat. Pharmacodyn.*, xx, 1910, p. 205.)

NaCl	8·00 grm.
KCl	0·20 ,,
$CaCl_2$, $6H_2O$	0·395 ,,
$MgCl_2$, $6H_2O$	0·213 ,,
NaH_2PO_4, $4H_2O$	0·05 ,,
$NaHCO_3$	1·0 ,,
Glucose	1·0 ,,
Aq. dest.	1000·0 c.c.

Weigh each salt on an analytical balance or make up concentrated solutions and add the calculated volume of each solution to the remaining distilled water. All salines containing $NaHCO_3$ must be sterilised by a Berkefeld or Seitz filter, not by autoclaving. The best time to sterilise the solution is immediately after mixing. Then test the *p*H which should be between 7·6 and 7·8. Loss of CO_2 through faulty stoppers will render it more alkaline.

Pannett and Compton saline. (See PANNETT and COMPTON, *Lancet*, i, 1924, p. 381.)

This saline is widely used in England and has the advantages of withstanding autoclave sterilisation and of rapid adjustment of its *p*H. Make up *Solution A :*

NaCl	8·0 grm.
KCl	0·42 ,,
$CaCl_2$	0·20 ,,
Aq. dest.	100·0 c.c.

Solution B :

Na_2HPO_4, $12H_2O$	0·43 grm.
NaH_2PO_4, $4H_2O$	0·043 ,,
Aq. dest.	100·0 c.c.

Take 88 c.c. of double distilled water and add 8 c.c. of solution A. Measure 4 c.c. of solution B into a small flask and autoclave the separate solutions. After cooling add B to A with a sterile pipette. The proportion of acid phosphate may be varied slightly to give a required *p*H. See NORFIELD (*Lab. Journ.*, vi, 1930, p. 320) for variation of this formula.

Locke-Lewis solution. (See LEWIS and LEWIS, *Anat. Rec.*, v, 1911, p. 277.)

This saline has been much used for culturing in a fluid medium without the addition of embryonic extracts or plasma.

Locke Solution :

NaCl	9·0 grm.
KCl	0·42 ,,
$CaCl_2$	0·24 ,,
$NaHCO_3$	0·20 ,,
Aq. dest.	1000·0 c.c.

For Locke-Lewis solution make up a 15 to 20 per cent. solution of chicken bouillon in Locke solution and add 0·5 per cent. dextrose.

Ringer Solution (used for heparin solutions). See page 731.

781. pH Determination of Salines and Extracts. Phenol red (pH range 6·8–8·4) is fortunately non-toxic to living cultures and withstands autoclave sterilisation. It is convenient to have ampoules containing about 1 c.c. of 0·5 per cent. sterile aqueous solution in stock. A set of standard buffers are also required for comparing the phenol red coloration of these and the saline solutions or extracts. If the saline is too alkaline add more sterile NaH_2PO_4 solution or bubble sterile CO_2 through it. Carrel adds a few drops of phenol red to the medium in flask cultures and so controls the pH of the culture approximately by adding appropriate mixtures of CO_2 and air to the sealed flask.

782. Preparation of Plasma. The important factors in preparing plasma, apart from complete sterility and speed are :—

(1) The prevention of contamination of the blood with tissue juices.

(2) Collecting the blood and later the plasma in contact only with paraffin wax or sterile oils.

(3) Chilling the blood as soon as possible after its withdrawal.

Fowl Plasma. When large quantities are required for routine work, bleeding from the carotid artery is to be preferred. Select a cockerel about one year old and starve for twenty-four to thirty-six hours, allowing it plenty of water. Anæsthetise the bird lightly, outside the operating room, and tie it on its back to a dissection board with the ventral surface of the neck conveniently orientated for operation. Remove the feathers from the neck and swab the skin and the remaining feathers with alcohol. Cover the body with a sterile cloth bag or with towels and bring it into the operating room. After making a longitudinal incision in the mid-ventral line of the neck, reflect the skin and clip its edges to sterile towels. Expose the carotid artery on one side only. The vessels lie close together in the mid-line just deep to the fascia covering the anterior neck muscles. First ligature the artery distally with silk or gut and pull it gently forwards into a small loop on which a loose proximal ligature should then be tied. Make a small incision between the two ligatures and immediately wash the cut vessel with sterile saline solution to remove tissue juices. Flood the artery and surrounding neck tissues with sterile olive oil or paraffin. A metal cannula, sterilised in oil, or

a paraffin coated glass cannula is then inserted into the artery and tied lightly with the proximal ligature. The blood flow is controlled by light pressure with the finger on the most proximal part of the exposed artery. After a few drops of blood have escaped the remainder may be collected in sterile, ice-cooled, paraffin coated tubes, fitted with corks or rubber stoppers. As each tube is half or three-quarters full, return it to a freezing mixture. The anæsthesia should be light throughout this procedure.

Centrifuge the blood at 2,500 revolutions per minute for eight to ten minutes, using ice in the centrifuge buckets in summer. The supernatant plasma is then removed with a paraffin coated pipette to paraffined storage tubes fitted with cork or rubber. After forty-eight hours in paraffin coated tubes the plasma will remain fluid even in clean glass tubes. It should always be stored in the refrigerator where it keeps liquid for at least a month or more. No anti-coagulant is necessary in preparing fowl plasma.

In an emergency, paper centrifuge tubes may be used. They should be immersed in paraffin wax at 120° C. and allowed to cool. If half an inch of sterile paraffin oil is placed in each glass or paper collecting tube, the blood remains sealed off from the atmosphere during centrifuging. In this way " true " plasma without loss of CO_2 and consequent change in pH is obtained.

About 50 c.c. of blood may be taken from a fowl if the wound is to be sutured and the bird allowed to recover for subsequent removal of its blood from the intact carotid artery on the other side. A rapid method of taking small quantities of blood from the heart of the fowl is described in detail by LEWIS (*Archiv. f. exper. Zellforsch.*, vii, 1928, p. 82).

783. Mammalian Plasma. Using ordinary laboratory equipment, an anti-coagulant is essential in the preparation of mammalian plasma. Heparin (see CRACIUN, *Bull. Johns Hop. Hosp.*, xxxviii, 1926, p. 327) is the most useful anti-coagulant, since it withstands autoclave sterilisation and does not appear to affect the growth of cultures. Fischer uses 0·1 per cent. heparin in Ringer solution, and Cameron recommends diluting this concentration, after sterilisation, with Tyrode solution, 1–5,000. Most workers prefer to expose the heart under aseptic conditions and to draw off the blood from the ventricle with a glass syringe. Either sterilise the syringe in paraffin oil and transfer the blood to a paraffin coated tube containing heparin (1 c.c. of 0·01 per cent. heparin is sufficient for one rat, rather more for a guinea-pig), or take up the heparin in a dry sterilised syringe before drawing the blood. The rest of the technique is exactly similar to that described for the fowl. Small amounts of blood can be obtained by heart puncture through the sterilised skin of the lateral thoracic wall with subsequent recovery of the animal.

Human blood is best drawn from the median basilic vein of the arm. FUCHS (*Arch. f. exper. Zellforsch.*, xiv, 1933, p. 334) has described a special centrifuge with which one can prepare mammalian plasma of all species without an anti-coagulant. It is not necessary to balance the buckets in this improved model and the speed attained throws down all the platelets in a very short time. For further methods of preparing mammalian plasma without anti-coagulants see BARONI, *Compt. rend. Soc. Biol.*, civ, 1930, p. 599.

For the *p*H determination in blood and plasma, see the methods of DALE and EVANS, *Journ. Physiol.*, liv, 1920, p. 167, and MARTIN and LEPPER, *Biochem. Journ.*, xx, 1926, p. 37.

Preparation of Serum. Mammalian plasma, clotted in a tube by the addition of a few drops of embryonic extract, readily yields serum after centrifuging, if the clot is broken up with a glass rod. Fowl plasma treated in this manner gives much less, but the yield is increased by shaking the clotted plasma in a flask with sterile glass beads and then centrifuging.

784. Embryonic and Tissue Extracts. Chick embryos of eight to ten days' incubation or mammalian embryos of approximately half the normal gestation period are the most suitable for making nutrient extracts. In the case of chick embryos the larger blunt end of the egg is tapped with forceps and the shell, forming the wall of the air chamber, removed without breaking the inner shell membrane. This membrane is then torn through with sterile forceps, the membranes and yolk sac are severed with scissors, and the embryo lifted out without touching the shell. Place the embryos in a sterile Petri dish with excess Tyrode solution and remove the fragments of membranes which tend to float in the extract after centrifuging. Some workers also remove the eyes to prevent contamination of the extract with pigment. After washing the embryos in two or three changes of Tyrode to free them as far as possible from blood corpuscles, transfer them to a dry Petri dish and mince them finely with curved scissors. The embryonic pulp should be taken up in short lengths of narrow-bore glass tubing, fitted with rubber teats for transference to the centrifuge tubes. Centrifuge the pulp for ten minutes at 3,000 revolutions per minute. This should be sufficient to throw down all embryonic cells, but as an extra precaution, the extract may be frozen solid with CO_2, or in a freezing mixture.

FISCHER (*Archiv. f. exper. Zellforsch.*, i, 1925, p. 122) has figured a simple apparatus for making tissue extracts which is very useful where large quantities are required. The adaptation of a glass syringe for this purpose is described by EARLE (*Archiv. f. exper. Zellforsch.*, xiii, 1932, p. 510).

The Concentrations of Embryonic Extract. The preparation of embryonic extract cannot be standardised very accurately, but

some attempt should be made to work with approximately constant dilutions of the embryonic juice in Tyrode solution. Fischer adopts the method of removing the concentrated juice after the first centrifuging and replacing it with a volume of Tyrode solution equal to that of the remaining pulp, which is then well mixed with a glass rod or pipette and returned to the centrifuge. The second extract is particularly suitable for hanging drop cultures of rapidly growing cells such as fibroblasts. A third, weaker extract, useful for maintaining organotypic growth in a so-called growth restricting medium, is prepared by repeating the process of adding another volume of Tyrode and re-centrifuging. The concentrated extract is used principally in varying dilutions of Tyrode solution for flask cultures. The worker must ascertain, by experiment, however, which type and concentration of extract is most suitable for the particular tissues to be cultivated and a clear description of the method of preparing the extract should be included in the account of tissue culture experiments.

Embryonic extract does not keep well even in the refrigerator and should be prepared fresh for each batch of cultures. Its growth promoting principle appears to be destroyed by heating over 56° C. Nevertheless heat inactivated extract is sometimes used where it is desirable to suppress the growth of connective tissue cells and to favour the migration of epithelia (see DOLJANSKI, *Arch. f. exper. Zellforsch.*, xi, 1931, p. 261). Berkefeld sterilisation of embryonic extracts is not to be recommended.

785. Tissue Extracts. No extract from adult tissues and organs has been found to possess the same power of stimulating the growth of tissue cultures as embryonic extract; but, in special instances, better initial growth of an organ has resulted if the medium contains also the juice of that organ (see AMOROSO, *Archiv. f. exper. Zellforsch.*, xii, 1932, p. 274). A useful table indicating the *p*H of tissue extracts from a wide variety of laboratory animals should also be consulted (KUTSCHERENKA and SOLOWIEW, *Biol. Gen.*, ii, 1920, p. 523).

For special growth-promoting tissue extracts, see CARREL, *Journ. Exper. Med.*, xxxvi, 1922, p. 385 (leucocytes), and for artificial substitutes for embryonic extract, consult CARREL and BAKER, *Journ. Exper. Med.*, xliv, 1926, p. 503.

786. Preparation of Cultures. If possible, the setting up of cultures in rooms used for other purposes should be avoided. The use of glass boxes of various designs with holes for inserting the arms are a hindrance to speed and to the fine dissection of small embryos under sterile conditions. The reader will find several excellent plans for laying out a tissue culture room in the textbooks recommended at the end of the chapter. It should be noted that the simplest possible arrangement of the apparatus combines to give speed and accuracy as well as complete sterility. Select a

room without unnecessary furniture and no dust-collecting ledges. Ensure that the room is free from air currents and not overheated by direct sunlight. Where a considerable number of cultures are to be maintained, it may be convenient to provide the culture room with taps through which superheated steam can be released just before the room is used. Cover the tissue culture table with a sterile black cloth (casement cloth or sateen). A few square blocks of wood about 1 inch thick, beneath the table cloth, are useful as platforms for delicate manipulations. A small Bunsen burner should be handy for the rapid re-sterilisation of instruments and the flaming of flask and tube apertures. A pot of paraffin wax containing about 25 per cent. of vaseline for sealing hanging-drop cultures is kept on a warm plate. Stands for sterile centrifuge tubes in which pipettes may be rested free from bacterial contamination are particularly useful (see Fischer, Gewebezüchtung, Müller and Steinicke, München, 1930). The operator should work in a sterile gown and wear a surgical mask covering the nose, mouth and hair.

Four chief methods of explanting tissues are now in general use, and many minor variations of these have been described.

787. The Hanging-drop Technique. Small explants are set up on a coverslip, which is inverted over a cavity slide and sealed with paraffin wax or vaseline. If fluid medium is used the culture is kept resting on the coverslip and not hanging from its under surface, as in the solid media cultures. These preparations are suitable for cytological work with a limited number of sub-cultures.

Lay out six to twelve sterile coverslips on a sterile black tile or sheet of xylonite and cover with a large Petri dish. Prepare an equal number of cavity slides by painting a broad band of wax just outside the circumference of the cavity. The thickness of this wax layer will help to raise the inverted culture well away from contact with the cavity of the slide, particularly if the cavity is shallow. Cover these slides with a Petri dish.

Dissect out the tissue to be cultivated and place it in a small amount of Tyrode solution in a fresh cavity slide. Wash the tissue free from blood corpuscles which degenerate if carried over into the medium, and inhibit growth. Cut the tissue into small explants of equal size with iridectomy knives. The explants should be about 1 to 1·5 mm. in diameter. Do not traumatise the tissue by tearing it with blunt knives or by squeezing it with forceps. If all conditions are favourable the explants will remain translucent like the living, intact embryonic tissues. Sometimes one finds that explants become opaque and whitish in appearance. This striking protoplasmic change appears to be due to injury and possibly to incorrect *p*H of the saline solution. If a culture is similarly injured by blunt knives or by undue stretching, it will also show this change instantaneously. Cultures of the opaque

type of explant do not grow so well as those made from the translucent tissue. They tend to become necrotic in the centre of the explant; a phenomenon which can be avoided entirely in small explants if due care is taken at the outset.

Place 1 drop of plasma, taken up in a fine sterile pipette, in the centre of each coverslip, and transfer an explant to each with iridectomy knives. Add 1 drop of embryonic extract to each, and with the utmost speed, mix the two media, spreading it evenly over the surface, but not as far as the edge of the coverslip. This can be done quite easily with iridectomy knives, and usually there is time to mix twelve cultures before the plasma clots. In summer the plasma clots rather more quickly. The evenness with which the medium is spread before clotting has an important effect on the distribution of the growth at the circumference of the explant. Invert a waxed cavity slide over the coverslip and, with pressure, it will adhere to the wax. The whole preparation is then inverted and rapidly sealed with the wax mixture.

Incubate cultures of fowl tissues at 38·5° to 39° C. and mammalian tissues at 37·5° C.

Sub-culturing. Every forty-eight hours the explants in hanging-drop cultures should be washed in Tyrode solution and fresh medium added. The wax sealing the culture is carefully removed with a hot scalpel and the coverslip taken up with sterile forceps and placed, culture upwards, in a Petri dish, remembering that only the region of the coverslip adjacent to the medium is sterile. Four tangential cuts across the periphery of the zone of new growth are made with iridectomy knives, and the culture lifted out with a minimum of the old medium adhering to it. The cells which have migrated nearest to the extreme edge of the medium are usually degenerate and are in this way also discarded. Wash the explants in Tyrode solution for at least ten minutes, and cut them into smaller fragments if necessary. The cultures are then treated as original explants prepared with fresh coverslips and media.

Maximow (*Contrib. Embryol. Carneg. Instit.*, xvi, 1925, p. 47) has devised a useful variation of the hanging-drop technique where it is desirable not to disturb the explant and new growth during sub-culturing. A small sterile coverslip, holding the culture, is fixed with a drop of sterile saline to the surface of a larger coverslip. Both are inverted over a large cavity slide. The inner coverslip remains sterile and may be detached for immersion, with its explant, in Tyrode solution prior to renewing the medium and re-sealing. Coverslip cultures may also be placed in small numbers in a Petri dish containing moist filter paper, with a large watch-glass inverted over them in addition to the cover of the Petri dish itself (see Barta, *Archiv. f. exper. Zellforsch.*, iv, 1927, p. 600). Coverslips of clear sheet mica are useful for the intermediate

passages of a culture when oil immersion observations are not required. Plasma cultures are more easily cut free of old medium on a mica surface. Afterwards the final sub-culturing can be made on glass coverslips for permanent stained preparations.

788. Flask Cultures. As many as six small explants can be grown in a single flask, and the larger volume of medium and the ease with which the whole preparation can be washed and the medium renewed, makes this technique suitable for histiotypic growth over long periods of time. The simple pattern of Carrel flask consists of a circular vessel with horizontal upper and lower surfaces, 3·5 to 5 cm. in diameter, and 1·25 cm. in height. A single tubular arm, 3 cm. long and 1 cm. in diameter leads into the flask at an inclined angle. This is fitted with a rubber cap made from a teat or with a special rubber stopper. A second arm placed opposite to the first allows greater freedom in manipulating the explants.

For the 3·5 cm. flask the following media are recommended by Fischer. Introduce 2·5 c.c. of plasma, 5 c.c. Tyrode solution and 1 drop of concentrated embryonic extract (from the first centrifuging) into the flask. Take up the explants in a pipette and arrange them at equal distances in the medium before it clots. Carrel prefers less solid medium and proceeds as follows: 0·5 c.c. plasma and 1·5 c.c. of 5 per cent. embryonic extract in Tyrode solution are placed in the flask with the explants. After one hour in the incubator, 1 c.c. of extract is added. For fibroblasts and epithelia he recommends 0·75 c.c. Tyrode solution containing 0·25 c.c. embryo extract. For leucocytes, 1 c.c. of equal volumes of Tyrode and serum. CAMERON ("Tissue Culture Technique," Farrar and Rinchart, New York, 1935) recommends the following procedure for mammalian flask cultures if homologous plasma and embryonic extract are unobtainable. Imbed the explants in a medium of chicken plasma, chick embryonic extract and Tyrode solution as described above. After clotting, fill the flask with Tyrode and incubate it for one hour. Remove the Tyrode and repeat the process of washing for one hour. Most of the foreign proteins should be eliminated by this washing. Homologous serum, Tyrode solution and embryonic extract (chick) can then be added for the final medium.

Washing the Flask Cultures and Renewing the Medium. This is done without disturbing the explants unless they have grown to inconvenient sizes. Using a length of fine platinum tubing which is repeatedly sterilised by heating in the Bunsen, and is attached by rubber tubing to an exhaust pump, the surplus medium is sucked from the flask and excess Tyrode solution added. After half an hour in the incubator the Tyrode solution is withdrawn with the suction apparatus and fresh medium, such as Tyrode and embryonic extract or serum, is pipetted into the flask.

Other Types of Flasks. The walls of the ordinary Carrel flask are too thick for oil immersion observation of the culture. Recently Carrel has devised a micro-flask of similar shape, 3 cm. in diameter with the horizontal surfaces remarkably flat and of average coverslip thickness. Another type of flask is provided with a large hole in its base over which a circular coverslip can be accurately sealed. Oil immersion observations are easily made with both these types of flasks.

McJunkin (*Archiv. f. exper. Zellforsch.* iv, 1927, p. 122) describes a simple method for making ordinary flasks from test-tubes. As the clotted medium containing the explants is not easily removed from Carrel flasks without breaking them, Doljanski (*ibid.*, xiii, 1932, p. 717) has invented a particularly useful pattern which has the shape of a Carrel flask, but with its base consisting of a detachable circular glass plate. This plate fits closely to a ground basal rim on the flask, and is sealed to it by a thin film of egg albumen.

789. Cultures in Watch Glasses. This method has been used with great success at the Strangeways Laboratory, Cambridge. Larger explants are imbedded on the surface of a large plasma clot in a watch-glass and bathed in very dilute embryonic extract which restricts the uncontrolled growth of migrating cells. The whole is then sealed in a Petri dish containing moist cotton-wool. Organotypic growth of entire early embryos, embryonic organs and skeletal fragments is best obtained by this technique.

790. The Continuous Irrigation Technique of de Haan (Durchstromungsapparat). This is a more elaborate arrangement whereby the culture is constantly irrigated by fresh nutrient medium. More recently the apparatus has been modified by including devices for saturating the fluid medium with a controlled gas mixture and hence adjusting the *p*H. Space does not permit the detailed description of this apparatus and its several modifications; see de Haan, *Bull. d'Histol. Appl.*, iv, 1927, p. 293; Suy, *ibid.*, viii, 1931, pp. 210, 294; Julius, *Acta brev. Nederl.*, ii, 1932, p. 69.

791. The *p*H and Gaseous Environment of the growing culture. Mention has already been made of Carrel's simple method of adding phenol red to the culture medium and introducing CO_2 in varying concentrations into the flask for control of the *p*H. More elaborate methods are given by Earle (*U.S. Pub. Health Rept.*, xlvi, 1931, p. 1997 (hanging-drop cultures) and p. 2668 (flask cultures)). Cf. also Earle (*Archiv. f. exper. Zellforsch.*, xvi, 1934, p. 116). See Schade (*ibid.*, xv, 1934, p. 121 for the cultivation of tissues in a constant gas mixture.

The roller-tube apparatus described by Gey (*Amer. Journ. Cancer*, xvii, 1933, p. 752), consisting of a revolving drum containing a series of glass culture tubes, provides for the constant

circulation of a limited volume of fluid medium which can be changed at intervals of a few days. This new technique is specially to be recommended for the maintenance of large numbers of cultures for prolonged periods.

792. Cultures from Non-Sterile Tissues. Several workers have attempted to sterilise tissues before explantation by ultra-violet radiation. Light and all forms of radiation appear to have a harmful effect on living tissues and CHLOPKOW (*ibid.*, x, 1930, p. 299) recommends, for adult rabbit intestine, the repeated washing of explants in sterile saline solution (Ringer). He also suggests washing in 1 in 40,000 Rivanol solution.

793. The Fixation and Staining of Tissue Cultures. Permanent stained preparations of hanging-drop cultures are readily made from the original coverslip on which the tissue has been growing. Flask cultures are generally sectioned in series. Cultures in fluid media are best fixed in the vapour of 2 per cent. osmic acid, iodine or formol, followed by almost any appropriate staining. Cultures in solid plasma medium require more care in staining owing to the strong affinity of fibrin for most histological and cytological stains. When the last sub-culture is made, the medium should be spread as thinly as possible on the coverslip before it clots. Washing in Tyrode solution for half an hour in the incubator before fixation may be followed by almost any fixative and stain without obscuring the cells in darkly stained plasma. With practice it is possible to dissect away the peripheral layers of a fixed plasma clot without damaging the culture, by using fine glass micro-needles and a dissecting microscope. This technique is useful when highly mordanting fixatives such as Regaud or Champy's fluids are employed.

Fixation for ten minutes in Zenker-formol and staining with Heidenhain's iron hæmatoxylin is particularly successful for routine preparations. WALLBACH (*Archiv. f. exper. Zellforsch.*, x, 1931, p. 383) recommends Heidenhain's "Susa" fixation for vitally stained cultures. Giemsa and most other blood stains may be used and the silver impregnation of fibres by routine methods can also be employed. The Feulgen reaction (see COWDRY, *Science*, lxviii, 1928, p. 138) stains the nuclei of cells in the zone of new growth and in the explant with a brilliance equal to that of sectioned tissues. The period of fixation for cultures can, in most instances, be considerably shortened and fixatives containing high concentrations of chromic acid and mercuric chloride are best diluted.

794. Serial Sectioning of Tissue Cultures. MAXIMOW (*Arch. f. mikr. Anat.*, xcvi, 1922, p. 494) has described a short celloidin imbedding technique for cultures. Care should be taken in paraffin imbedding to expose the preparation only for very short periods in frequent changes of wax. Prolonged immersion in

melted wax causes great shrinkage of the plasma clot. Half an hour's imbedding in paraffin is quite sufficient for small cultures.

For detailed information on the technique of tissue culture consult the following text-books:

CAMERON, G., "Essentials of Tissue Culture Technique," Farrar & Rinehart, N. York, 1935; CRACIUN, E. C., "La culture des tissus en biologie experimentelle," Masson et Cie, Paris, 1931; DEMUTH, F., "Praktikum der Zuchtung von Warmblutergewebe in vitro," Müller & Steinicke, München, 1930; EPHRUSSI, B., "La culture des Tissus," Gauthiers Villars, Paris, 1932; ERDMANN, R., "Praktikum der Gewebeflege oder Explanation besonders Gewebezüchtung," Julius Springer, Berlin, 1930; FISCHER, A., "Gewebezüchtung," Müller & Steinicke, München, 1930; LEVI, G., *Explanation, besonders die Struktur und die biologischen Eigenschaften der in vitro gezuchteten Zellen und Gewebe. Ergebn. Anat. u. Entwick.*, xxxi, 1934, s. 125; NORFIELD, V. C., *The Technique of Tissue Culture in vitro, Lab. Journ.*, vi, 1930, pp. 320—329, 366—380; STRANGEWAYS, T., "The Technique of Tissue Culture in vitro," W. Heffer & Sons, Ltd., Cambridge, 1924.

CHAPTER XXXIII

EMBRYOLOGICAL METHODS

795. New Advances. In nearly every case the newest advances in ordinary embryological technique * are constituted by the improvements in fixation reported in the new sections on Cytology, Glands, and in Imbedding (§ 124). It would be a great mistake for observers to consider that fixation and staining methods, such as those of Champy-Kull, Kopsch, or Flemming-without-acetic acid, and iron hæmatoxylin, are of no concern to embryologists. For instance, amphibian embryos, such as those of Triton (Molge) prepared by Champy-Kull's method, are extremely beautiful and instructive for study, for not only does one procure cytological perfection, but also a staining which is polychromatic. For the study of invertebrate embryology, the mitochondrial methods open up a new field for research.

The reliability of many of the new neurological methods (see §§ 977 to 1107) has been brought to a state which should now induce embryologists to devote even rare material for preparation, and to use the neurological methods much more widely than at present is the case.

In § 648 is a special treatment of the study of fats, the newer methods for which can readily be used for embryological studies. In § 666 is a section on "Glycogen, Iron and Copper, etc." In § 778 is a chapter on the "Tissue Culture" methods. In § 739 to § 775 is a report on "Intravital Staining." The newer imbedding methods will be useful (§ 124).

796. Artificial Fecundation. This practice, which affords the readiest means of obtaining the early stages of development of many animals, may be very easily carried out in the case of the amphibia Anura, Teleostea, Cyclostomata, Echinodermata, and many Vermes and Cœlenterata.

In the case of the Amphibia, both the female and the male should be laid open, and the ova should be extracted from the uterus and placed in a watch-glass or dissecting dish, and treated with water in which the testes, or, better, the vasa differentia, of the male have been teased.

Females of Teleostea are easily spawned by manipulating the belly with a gentle pressure; and the milt may be obtained from

* Save the techniques of the Ross Harrison and Spemann schools, which seem perhaps outside the scope of this work.—Eds.

the males in the same way. (It may occasionally be necessary, as in the case of the Stickleback, to kill the male, and dissect out the testes and tease them.) The spermatozoa of fish, especially those of the Salmonidæ, lose their vitality very rapidly in water; it is therefore advisable to add the milt immediately to the spawned ova, then add a little water, and after a few minutes put the whole into a suitable hatching apparatus with running water.

Artificial fecundation of Invertebrates is easily performed in a similar way. For methods of artificial *Parthenogenesis* see Harvey, *Biol. Bull. Wood's Hole*, 1910, p. 269.

797. Superficial Examination. The development of some animals, particularly some invertebrates, may be to a certain extent followed by observations of the living ova under the microscope. This may usefully be done in the case of various Teleosteans, such as the Stickleback, the Perch, *Macropodus*, and several pelagic forms, and with *Chironomus*, *Asellus aquaticus*, Ascidians, *Planorbis*, many Cœlenterata, etc.

Some ova of insecta and Arachnida which are completely opaque under normal conditions become transparent if they are placed in a drop of oil; if care be taken to let their surface be simply impregnated with the oil, the normal course of development is not interfered with (Balbiani).

798. Fixation. Osmic acid, employed either alone or in combination with other reagents, is an excellent fixing agent for small embryos, but not at all a good one for large ones. It causes cellular elements to shrink somewhat, and therefore brings out very clearly the slits that separate germinal layers, and any channels or other cavities that may be in course of formation.

In virtue of its property of blackening fatty matters, myelin amongst them, it is of service in the study of the development of the nervous system.

Chromic acid is indispensable for the study of the external forms of embryos; it brings out elevations and depressions clearly, and preserves admirably the mutual relations of the parts; but it does not always preserve the forms of cells faithfully, and is a hindrance to staining in bulk.

Picric liquids have an action which is the opposite of that of osmic acid; they cause cellular elements to swell somewhat, and thus have a tendency to obliterate spaces that may exist in the tissues. But notwithstanding this defect, the picric compounds, and especially Kleinenberg's picro-sulphuric acid, are amongst the best of embryological fixing agents.

Schridde (*Zeit. wiss. Mik.*, xxvii, 1910, p. 362) finds Orth's "Formol-Müller" in general the best fixative. Fix for not more than twenty-four hours, and pass through graded alcohols (twenty minutes in each) into absolute (one to two hours), cedar oil, xylol, and paraffin.

RABL (*Zeit. wiss. Mik.*, xi, 1894, p. 165) recommends for embryos of Vertebrates, and also for other objects, his platinic sublimate, § 81. This serves for a large number of blastoderms and *young* embryos (Pisces, Amphibia, Aves, Mammalia). *Advanced* embryos of Teleostea ought to be fixed in the *warmed* mixture, in order to avoid rupture of the muscles and shrinkage of the chorda.

Some of his best results were obtained by a *not too prolonged* fixation in a mixture of

Platinic chloride, 1 per cent. solution	1 volume.
Picric acid, saturated aqueous .	2 volumes.
Distilled water	7 ,,

RABL's picro-sublimate mixture has been given, § 75. It is recommended especially for somewhat advanced embryos, such as embryo chicks from the third or fourth day, and other embryos of a similar size.

BOVERI (*Verh. Phys. Med. Ges. Würzburg*, xxxix, 1895, p. 4), in order to imbed and cut together numbers of ova of Echinoderms, wraps them in pieces of sloughed epidermis of *Cryptobranchus* (of course, other Urodela will do). SOBOTTA (*Arch. mik. Anat.*, l, 1897, p. 31) takes pieces of amnion of Mammalia.

SANZO (*Zeit. wiss. Mik.*, xxi, 1904, p. 449) describes an automatic apparatus for fixing material at definite stages.

799. Removal of Albumen. The thick layers of albumen that surround many ova are a serious obstacle to the penetration of reagents. CHILD (*Arch. Entwickelungsmech.*, ix, 1900, p. 587) gives the following as of very general applicability. After fixation (in any way except with chromic acid) the ova are brought through graduated alcohols up to that of 80 per cent., in which they are hardened. They are then brought down again through successive alcohols into water acidified lightly with any acid (except chromic acid), and the albumen is found to become transparent and dissolve.

800. Reconstruction of Embryos from Sections. To facilitate the study of series of sections, recourse may be had to graphic or plastic reconstruction of the objects.

In simple cases it may be sufficient to adopt the plan described by SCHAFFER (*Zeit. wiss. Mik.*, vii, 1890, p. 342). Careful outlines of the sections to be constructed are drawn on tracing paper with the aid of the camera lucida, superposed, and held up against the light for examination by transparence. VOSMAER (*Anat. Anz.*, xvi, 1899, p. 269) draws on plates of celluloid, and sets them up in a rack for examination. KERR (*Quart. Journ. Mic. Sci.* xlv, 1902, p. 1) draws on plates of ground glass which he afterwards superposes and makes transparent by oil of cloves run in between them. PENSA (*Zeit. wiss. Mikr.*, xxvii, 1910, p. 48) takes sheets

of lithographic gelatin. WOODWORTH (*Zeit. wiss. Mik.*, xiv, 1897, p. 15) proceeds as follows: (1) Draw an axial line of the length of the object multiplied by the magnification employed. (2) Measure with a micrometer the greatest diameter of each section. (3) Plot these diameters down transversely on the axial line at distances corresponding to the thickness of the sections multiplied by the magnification. (4) Join the extremities of these diameters; this will give you an outline of the object. (5) Measure off on each section the nearest and farthest limits (from the margin) of the organs to be filled in, and plot them down on the transverse lines (3), and join the points as before, *i.e.* from section to section; this will give you the outlines of the organs.

This process is best applicable to reconstruction from transverse sections, but it can be applied to reconstruction from sections in any plane if the object can be provided with a plane of definition at right angles to the plane of section. This may be established by cutting off one end of the object, or the like (see also *Orientation*, §§ 162, 164).

To make a simple *plastic* reconstruction, camera drawings (or photographs) of the sections (all made at the same magnification) are pasted on pieces of cardboard of a thickness equal to that of the sections multiplied by the magnification employed. Then the parts of the drawings representing the cavities of the objects are cut out with a knife or fretsaw, cutting through the cardboard; and the pieces of fretwork thus obtained are pasted together.

Many useful modifications of this method have been devised. Cardboard is rather hard to cut, and not conveniently got of the required thickness. The late Professor Arthur Thompson, of Oxford, used numbers of sheets of blotting paper to the required thickness, soaked in beeswax; this makes a very tough substance, and the models, when made, can be handled without chance of injury; other workers use beeswax plates alone, drawing the outline with some sharp instrument and cutting out with a hot knife.

Mr. Pittock, of the Embryological Laboratory, University College, London, uses a modification of K. Peter's method (*vide infra*). Rather thin paper is used for drawing the outline of the object. In this laboratory (Professor J. P. Hill), special rolls of paper are used, so that the diagram of each of hundreds of sections may be safely rolled up in order till wanted. A large flat stone is used for the manufacture of the wax plates, with two brass gauges of the required thickness placed at a distance which will accommodate in between them the square of paper with the drawing. Instead of treating the paper with turpentine, according to Mr. Pittock's method, the drawing is rapidly floated over the surface of a dish of water, drawing side down, then laid upon the stone, between the metal gauge, and the superfluous moisture smoothed

off with a sheet of blotting paper. The melted wax is poured on to the paper, and a heated metal roller, passing over the metal gauge, leaves just the required amount of wax on the paper. The latter easily peels off the surface of the stone.

For more elaborate processes of plastic reconstruction (very complicated and seldom necessary) see BORN, "Die Plattenmodelliermethode," in *Arch. mik. Anat.*, 1883, p. 591, and *Zeit. wiss. Mik.*, v, 1888, p. 433; STRASSER, *ibid.*, iii, 1886, p. 179, and iv, pp. 168 and 330; KASTSCHENKO, *ibid.*, iv, 1887, pp. 235—6 and 353, and v, 1888, p. 173; SCHAPER, *ibid.*, xiii, 1897, p. 446; ALEXANDER, *ibid.*, p. 334, and xv, 1899, p. 446; PETER, *ibid.*, xxii, 1906, p. 530; BORN and PETER, *ibid.*, xv, 1, p. 31; and *Verh. Anat. Ges.*, xiii, 1899, p. 134; JOHNSTON, *Anat. Anz.*, xvi, 1899, p. 261; FOL, *Lehrb.*, p. 35 or *previous editions*; BROMAN, *Anat. Hefte*, xi, 1899, p. 557; PETER, "Die Methoden d. Rekonstruction" (Fischer, Jena, 1906); SCHÖNEMANN, *Anat. Hefte*, xviii, 1901, p. 117; GAGE, *Anat. Record*, i, 1907, p. 167; NEUMAYER, *Festschr. f. Kupffer*, 1899, p. 459; MARK, *Proc. Amer. Acad. Sci.*, xlii, 1907, p. 629 (electric wax-cutter for cutting out plates).

HILL (*Bull. Johns Hopkins Hosp.*, xvii, 1906, p. 114) finds that embryos of mammalia taken from 95 per cent. alcohol and put into caustic potash of 1 per cent. become so transparent that they can be studied without cutting and reconstructing.

MAMMALIA

801. Times for Early Development. The entry of the sperm into the egg of the mouse takes place from six to ten hours after copulation (SOBOTTA, *Arch. mikr. Anat. Bd.*, 45). The pro-nuclei stage of fertilisation is found from eighteen to twenty-two hours, two-cell stage twenty-six hours, four-cell, fifty hours, eight-cell, sixty hours after copulation: the egg remains in the tube about eighty hours. J. A. LONG and E. L. MARK (*Contrib. Zool. Lab. Museum, Harvard, Carneg. Inst. Wash.*, No. 142, 1911) find in the mouse that ovarian eggs within fifteen or sixteen hours after parturition have formed the first maturation spindle. Fertilised eggs are obtained from animals killed between twenty-three and thirty-one hours *post partum*. The time required for the spermatozoa, after introduction into the uterus (either artificially or by coitus) to reach the eggs in the first part of the oviduct varies from four to seven hours in mice inseminated about the same number of hours *post partum*. To obtain free eggs for study, Mark and Long kill mice fourteen to seventeen hours after parturition, the ova being found in a fold of the oviduct.

In the rat the eggs are found in the oviduct about 18·7 hours and ovulation occurs in less than eighteen hours *post partum*.

In the rabbit the pro-nuclei stage of fertilisation occurs about fourteen hours, in the guinea-pig twenty-two to twenty-four hours after copulation (SOBOTTA). The rabbit's egg, like that of the guinea-pig, remains about eighty hours, the dog's egg eight to ten days in the tube (RÖTHIG, *Embryol. Technik*).

Condition of Ovary as Index to Pregnancy. On opening the body cavity of a mammal, first of all examine the ovary. By so doing one can estimate roughly the time that has elapsed since the discharge of the ovum or ova. Prominent stigmata or areas with a blood-clot centre indicate recent ovulation while a smooth surface of yellowish appearance indicates a corpus luteum, which means that some time has elapsed since ovulation.

802. Isolation of the Eggs and Early Stages.* The tubæ and uterus or uteri are dissected out and treated in one of two ways: either the isolated tuba after straightening is washed out from the funnel opening with warm salt solution, or with some fixative like formalin or weak osmic acid, or on the other hand the whole length of the tube is laid open and spread out with a scalpel or sharp scissors and needles, and the eggs are looked for under a dissecting microscope.

If the method of washing out is adopted, it is best to use a good rubber bulb attached to a glass tube which has been drawn out finely enough to pass into the oviducal opening. Kölliker used Müller solution or weak osmic acid for injection, collecting the fluid in a series of watch-glasses; J. P. Hill uses solid crystal dishes, which can easily be examined under a stereoscopic binocular microscope. As a fluid for washing out Hill's picronitric osmic (*vide infra*), weak formalin, or weak osmic acid are probably as good as anything. The success of this injection method depends on the amount of mucus in the tuba and on the condition of the folds in its mucosa; if the eggs are not found after the injection, the walls of the tube may be opened up with scissors and the lining scraped away with a small scalpel; the mucus thus procured may be diluted with a little indifferent fluid and examined on a slide under the microscope. Both operations of injection or of opening the tuba may succeed with comparatively large animals like the rabbit and dog. It is practically impossible to slit open the tuba of the cat.

In cases where the subject is small, as, for instance, the mouse, it is necessary to preserve the whole oviduct and use a fixative sufficiently penetrative to act quickly. Even with the guinea-pig the lumen of the tube is so small that it is difficult to remove the ova; we consider that attempts to press out the contents of the tubes are dangerous. In such cases it seems better to cut the tube into lengths with a razor and to fix whole (*vide infra*). Bischoff in his study on the guinea-pig (Giesson, 1852), and Ballowitz (*Arch. Anat. Physiol.*, 1883) both resorted to the method of squeezing out the contents of the tubes.

When found the ova are picked up with the point of a cataract needle or a scalpel, on a piece of black paper cut to a point, or with a pipette, and either examined fresh in the peritoneal fluid

*** See Allen and co-workers, Contrib. Embryol. Carneg. Inst. Wash. 1934.**

or blood serum of the animal, or in Kronecker's or other artificial serum media, or better fixed immediately.

Van der Stricht method of obtaining Ova from Fallopian Tube of Dog. Dissect out tube and stretch straight. Divide across into two parts. Then with gentle pressure from scalpel, squeeze along the length of the segment, so as to express the contents on a clean slide. A drop of viscid fluid exudes in which should be the ova. Fix by osmic vapour method, by inverting over mouth of osmic bottle. The fluid will coagulate and the block may be cut and transferred to the desired fixative.

In the case of a large animal such as the rabbit, the same doe may be made to serve for two observations, at some hours' or days' interval. A longitudinal incision of 8 to 10 centimetres' length is made on the medial or a lateral line of the abdomen; an assistant keeps the intestines in their place; a ligature is placed at the base of one of the uterine cornua, beneath the neck, and a second ligature around the mesometrium and mesovarium. The ovary, the tuba, and the cornu of that side are then detached with scissors. The abdomen is then closed by means of a few sutures passing through the muscle-layers and the skin. The animals support the operation perfectly well, and the development of the ova of the opposite side is not in the least interfered with. When it is desired to study these the animal may be killed, or may be subjected to a secondary laparotomy if it be desired to preserve it for ulterior observations. This method, however, cannot be carried out in England without a licence.

This procedure was also adopted by Hartmann in his study on Didelphys (*vide infra*).

803. Fixation of the Isolated Ova. These can be fixed in a chrome-formalin fluid of some kind: Müller-formol, Helly, Zenker-without-acetic acid and formol are indicated. Eggs may be left in one of these fluids overnight, then washed in distilled water and transferred either to 1 per cent. OsO_4, or to some chrome-osmic fluid, this to preserve the fat. The chrome fixation will form insoluble compounds with lipoids, but less so with fats of the type of olein. It seems likely that the fixation technique of Champy-Kull, of Schridde and of Murray (see §§ 691 *et seq.*) will be of great value.

For a study of the Golgi elements the methods of Cajal and Da Fano and of Ludford are worthy of trial, but rather more difficult to work than chrome-osmic or chrome-formol techniques. Where there may be a difficulty of penetration chrome-formol fluids will be found better than chrome-osmium. A perusal of the sections on Mitochondria and Golgi apparatus will provide suggestions for the treatment of the early stages in mammalian development. Van Beneden (*Arch. de Biol.*, 1880, p. 149) brings the living ovum into a drop of 1 per cent. OsO_4 on a slide, and thence into a solution of Müller. After an hour the liquid is changed, and the whole is put into a moist chamber, where it remains for two or three days. It is then treated with glycerin of gradually increasing strength,

and at last mounted in pure glycerin acidified with formic acid. We are inclined to believe that the Champy-Kull or Regaud fixation (the latter with a post-osmication) would be much superior to the above method, that is, for sectioning.

Many authors have used picro-nitric, picro-sulphuric, picro-formol with or without corrosive, chromic-acetic acid, Flemming and Hermann, and so on, but one cannot help thinking that the more modern and logical fixation methods will be better. This seems borne out by the late work of LAMS (*Arch. de Biol.*, t., xxiii), and LEVI (*Arch. f. Zellf.*, xiv.)

J. P. HILL (*Quart. Journ. Micr. Soc.*, 1910) gives the formula of a "Marsupial mixture" for fixation of ova and blastocysts of Marsupials. This fluid is made by adding to 96 c.c. of Mayer's picro-nitric, 2 c.c. of 1 per cent. OsO_4. Two c.c. of glacial acetic acid may be added, but the picric acid is sufficiently penetrative without the addition of acetic acid.

J. A. LONG (*Contrib. Zool. Lab. Museum Compar. Zool. Harvard*, 1912) describes an ingenious constant temperature box for working with fresh egg of mammalia. A circulation slide is also described in detail. So far J. A. Long has succeeded in keeping mice eggs alive and under observation for only twelve hours.

J. A. LONG and E. L. MARK (*op. cit.*) use a modified Zenker for their study on mouse eggs. They fix for from twenty to sixty minutes in a mixture of (A) 4 per cent. bichromate of potash. (B) 4 per cent. (aq. sol.) sublimate and 20 per cent. acetic acid. For use, mix equal portions of A and B. Wash out in warm water for twelve to fourteen hours, 70 per cent. alcohol and iodine twelve to fourteen hours, quickly dehydrate, clear in xylol and imbed in paraffin. Mark and Long's fixative appears to us (on paper at least) to be far too acid. It may be indicated for chromosome work.

804. Subsequent Treatment of Ova. After fixation the eggs or blastocysts should be brought into 30 per cent. alcohol and slowly upgraded to 90 per cent. alcohol: at this stage they may be stuck on pieces of liver or brain by MINCHIN's albumen method; the egg is placed on the liver and albumen is gently pipetted over it. The alcohol coagulates the albumen, and enables the object to be handled more easily. Another method used by J. P. HILL (*Quart. Journ. Micr. Science*, 1910) is to bring the ova into alcohol absolute and then into equal parts of alcohol absolute and ether. Then take a hand-cut section of liver or brain (which has been stored in absolute) place 1 drop of 0·5 per cent. solution of photoxylin (or celloidin) in equal parts of absolute alcohol and ether; then transfer the egg on a flat camel hair brush to this drop, and harden the object in 15 per cent. chloroform in 90 per cent. alcohol. Transfer to equal parts of absolute alcohol, xylol and chloroform. Then equal parts of chloroform and xylol, and imbed in paraffin wax.

The process of sticking the eggs to the hard cut liver or brain section should be carried out under a dissecting microscope.

805. Uterine Eggs. During the *fourth*, *fifth*, and *sixth* days after copulation the ova of the rabbit are free in the uterine cornua; they are easily visible to the naked eye, and may be extracted by the same manipulations as those of the tubes. After the sixth day they are at rest in the uterus, but have not yet contracted adhesions with the mucosa, so that they can still be extracted whole. At this stage the parts of the cornua where the ova are lodged are easily distinguishable by their peculiar aspect, the ova forming eminences of the size of a pea. The cornua should be cut up transversely into as many segments as there are eminences, care being taken to have the ova in the centre of the segments. You then fix each segment by means of two pins on the bottom of a dissecting dish, with the mesometrial surface downwards and the ovular eminence upwards. The dissecting-dish is then filled up with serum or liquid of Müller, or 0·1 per cent. solution of osmic acid, Bouin's fluid, Hill's fluid, Helly's fluid or 10 per cent. formol. See sections on " Cytology," §§ 691 to 714. With a small scalpel a longitudinal incision is made on the surface of the ovular eminence, not passing deeper than the muscular layer; the underlying uterine mucosa is then gently dilacerated with two pairs of small forceps, and the ovum set free in the liquid.

From the moment the ova have become adherent to the uterine mucosa they can no longer be extracted whole. The embryo being always situated on the mesometrial surface, the ovular eminence is opened by a *crucial* incision, and the strip of mucosa to which the embryo remains adherent is fixed with pins on the bottom of the dish. Ed. v. Beneden (see *Arch. de Biol.*, v, fasc. iii, 1885, p. 378) has been able by operating in this way in serum of Kronecker, and keeping the whole at blood temperature, to observe the circulation of the embryo for hours together. (If this be desired to be done, the crucial incision should not be too extended, so as to leave the terminal sinus intact.)

Retterer (*C. R. Soc. de Biol.*, 1887, p. 99) advises that for ova of the seventh day the segment of uterus containing them be opened *on the mesometrial surface*, for at that date no adhesion has yet been contracted with that side. By running in liquid of Kleinenberg by means of a pipette between the ovum and the free surface of the uterus, the ovum may be got away in the shape of a closed vesicle.

C. G. Hartmann (*Journ. Morph.*, 1916), in his study of the development of the opossum, used Carnoy's, Bouin's, Flemming's and Hill's fluids. He found Hill's " Marsupial mixture " a perfect fixing fluid for marsupial eggs. J. P. Hill now recommends leaving out the ascetic acid for delicate objects.

806. Blastoderms and Later Embryos. The routine methods of embryology apply here in general. Great care must be exercised

to avoid rough treatment caused by upgrading the object too quickly. The same remark applies even more particularly to clearing, which to get the best result should be done very gradually.

In order to bring out the outlines of blastoderm cells the living ovum may be brought into $\frac{1}{3}$ per cent. solution of nitrate of silver. After remaining there for half a minute to two minutes, according to the age of the vesicle, it is brought into pure water and exposed to the light. The preparations thus obtained are instructive, but blacken rapidly, and cannot be permanently preserved.

The blastodermic vesicle can be opened with fine needles, and the blastoderm washed, stained, or impregnated with gold, and mounted in glycerin or balsam.

For embryonic areas and more advanced embryos, refer to "Cytology," §§ 691—714. Kölliker recommends putting the ovum into 0·5 per cent. solution of osmic acid until it has taken on a somewhat dark tint, which happens in about an hour, and then treating it with successive alcohols for several hours. If the ovum be adherent to the uterine mucosa the portion of the membrane to which it is fixed should be left, stretched out with pins, in 0·1 per cent. solution of osmic acid for from four to six hours. The blastodermic vesicle can then easily be removed, and further treated as before. For sections Kölliker fixes with osmic acid. v. Beneden treats the ova for twenty-four hours with 1 per cent. solution of chromic acid, then washes well, and brings them through successive alcohols. Chromic acid has the advantage of hardening thoroughly the vesicle, and maintaining at the same time the epiblast cells perfectly adherent to the zona pellucida. v. Beneden also recommends the liquid of Kleinenberg. Henneguy writes that he frequently employs it for embryonic areas and embryos of various ages, always with excellent results. Fol's modification of the liquid of Flemming gives excellent results. For staining, Henneguy recommends borax-carmine, or Delafield's hæmatoxylin for small embryos; for large ones he found that his acetic acid alum-carmine was the only reagent that would give a good stain in the mass.

For sections imbed in paraffin, or double imbed.

807. On the Fixation of Whole Tubes. This may be done in Carnoy, Bouin or Helly. For rapidity of fixation, and faithfulness of preservation of cell aggregates Carnoy's fluid or preferably Sansom's modification of Carnoy are to be recommended. Chrome-formalin mixtures penetrate less readily, but often give fine results. Bouin's fluid we have found capricious. On the whole we think that warm Helly or Müller-formol as a preliminary fixation are to be recommended for small tubes. Regaud's or Schridde's methods should give efficient fixation (§§ 699, 702). Many workers have used the picric mixtures like picro-sulphuric and

nitric, and Kleinenberg's picric acid. Flemming's fluid has also been used.

In later stages of development some workers open the uterus under the fixative, or ligature one end of the organ and inject some fixing medium.

Corrosive formol mixtures have been much used for this purpose.

Neutral formalin of from 3 to 10 per cent. strength is often used for preserving later stages, after the uterus has been opened out. The advantage of this procedure from the cytological point of view is that any methods such as those of Regaud, Bensley-Cowdry, Sjövall, or formol-silver nitrate neurological techniques may subsequently be used. The chrome-picric or alcoholic acetic formol mixtures are not so suitable if one has cytological study in view.

808. On Clearing Mammalian Material. This is an important matter, because delicate embryos are easily shrunken up, or even not properly dealcoholised, by injudicious methods. J. P. Hill always clears in two stages. Dehydrated embryos are brought into cedar wood oil in which they are left overnight. See also methyl benzoate method (§ 177). The cedar wood oil is subsequently washed out in benzol for several hours according to size of object. Paraffin parings are then added to the benzol, contained preferably in a tube, and the latter is then left overnight uncovered on the top of the bath, and subsequently put into pure wax. This method ensures a gentle dealcoholisation, and an efficient imbedding. Neither cedar wood oil nor benzol cause the tissue to become brittle as happens often when one uses xylol or chloroform (see §§ 135 *et seq.*).

Imbedding. For embryological work of a critical character, especially with post-blastoderm stages, double-imbedding in celloidin and wax is generally indispensable (§ 190). It is only necessary to contrast serial sections of chick blastoderms prepared by this method with those obtained by wax imbedding alone to become convinced of the inability of the latter method to do complete justice to the details of the structure and relations of the embryonic tissues (Wilson and Hill, *Phil. Trans. Roy. Soc.*, 1907).

See also J. P. Hill (*Anat. Anz.*, Bd. xviii, 1900; *Quart. Journ. Micr. Science*, lvi, 1910); Hartmann (*Journ. Morph.*, xxvii, 1916). The latter recommends punching a hole in the side of larger blastoderms to facilitate penetration of dehydrating and clearing fluids. Weysse, *Proc. Amer. Acad. Arts and Sci.*, 1894, p. 285 (blastodermic vesicle of *Sus scrofa*); Sobotta, *Arch. mik. Anat.*, xlv, 1895, p. 15 (ovum of the Mouse; fixation in Flemming's weak mixture, sections stained with Benda's iron hæmatoxylin), and *Anat. Hefte*, 1 Abth., viii, 1897, p. 476 (Rabbit; fixation with liquid of Flemming or picro-sublimate with 2 per cent. acetic acid); Bonnet, *ibid.*, ix, 1897, p. 426 (Dog; fixation in sublimate); Selenka, *Stud. Entw. d. Thière*, Wiesbaden, 1883, p. 5, and

1887, p. 107 (picro-sulphuric acid for the mouse, and picric acid with $\frac{1}{16}$ per cent. of chromic acid for *Didelphys*); KEIBEL, *Morph. Arb.*, ii, 1893, p. 11 (*Sus scrofa*); NEUMAYER, *Festschr. f. Kupffer*, 1899, p. 458 (embryos of the sheep best fixed in Carnoy's acetic acid, alcohol, and chloroform, § 90); WINIWARTER, *Arch. Biol.*, xvii, 1900, p. 39 (mixture of 50 parts saturated sublimate in salt solution, 50 parts alcohol, 20 of 1 per cent. platinum chloride, and 5 of acetic acid); SPEE, *Encycl. mik. Techn.*, 1910, p. 353 (cornua of *Cavia* fixed for twelve to twenty-four hours in sublimate, and put into 0·5 per cent. osmic acid till light brown, then into iodine alcohol, in which the osmium is reduced); WIDAKOWICH, *Zeit. wiss. Zool.*, xciv, 1909, p. 243 (*Mus. rattus*, fixation in Zenker's mixture, or 2 parts of alcohol of 80 per cent. with 1 of formol; also instructions for dissection).

809. Injection and Clearing of Larger Embryos. A considerable amount of useful work has lately been carried out on embryonic blood and lymph vessels, and on the cerebro-spinal cavities, by micro-injection apparatus. A suitable injection medium is blown or forced into the vessels of an embryo, the latter is fixed and then dehydrated, and cleared by the Spalteholz method (*Über das Durchsichtigmachen von menschlichen und tierschen Präparaten, und seine theoretischen Bedingungen*, Leipzic, S. Herzel, 1911; 2 Aufl., 1914).

In an early stage in the formation of embryonic vessels and cavities the walls are thin and often ill-marked, and care must be taken not to burst through boundaries by excessive pressure. Very fine metal needles, or, better, finely drawn out glass cannulæ are used for injecting the specimens; the tube leading to the cannula is filled with the injection medium, which, by means of a rubber tube leading to the operator's mouth, is blown carefully into the perforated vessel or cavity. Or, one may use a rubber bulb either worked by hand, or placed on the floor and compressed by the foot. See E. M. GREGORY, *Anat. Record*, xi, 1917.

The injection media most commonly used are India ink, a saturated solution of Prussian blue, an aqueous suspension of lamp black, or silver nitrate (5 per cent.). The Prussian blue and India ink give about equal results, the blue clearing better, the ink being more opaque. The ink flows the better. Silver nitrate preparations are very beautiful and easy to analyse, but its caustic action prevents the finer vessels from filling. Lamp black tends to precipitate in fine flakes (CUNNINGHAM, *vide infra*). EVANS (*vide infra*), for cerebro-spinal spaces of pig embryos, injected potassium ferrocyanide, 0·5 grm., iron ammonium citrate, 0·5 grm., aq. dest., 100 c.c., and afterwards immersed the embryo for one to ten minutes in a 10 per cent. formaldehyde solution containing 1 per cent. HCl.

The embryo was then fixed in Bouin's fluid, but the Prussian blue faded after about a year.

SABIN (*vide infra*) and CUNNINGHAM, after India ink injection, fix in Carnoy's fluid, place in 80 per cent. alcohol, dehydrate in

graded alcohols, clear thoroughly, first in benzine (or benzol), and then in oil of wintergreen (Spalteholz). Embryos cleared by Spalteholz's method may later be imbedded from oil of wintergreen by transferring to half wax, half oil of wintergreen, and then pure wax. Tissues left in oil of wintergreen do not go brittle even after a year or two (Sabin).

Improved methods have recently been introduced by FRANKLIN P. REAGAN (*University of California Publications in Zoology*, 1926), who injects with undiluted filtered India ink, fixes in formalin of 4 per cent. to 10 per cent. with a trace of acetic acid for twenty-four hours if the material is to be dissected in water. The fixed tissue is somewhat clear, conveniently elastic. Carnoy's fluid shrinks the tissue and renders it tough. After fixation in formalin acetic, the embryo is washed for an hour in tap water, bleached in a mixture of 3 parts of water to 1 of hydrogen peroxide, which may produce a few bubbles in the tissue. The bubbles may be liberated by punctures in the skin. The bleaching process may last from a few minutes to a few hours. After bleaching, and removal of bubbles, the material is washed for a short time in water. It may then be returned to formalin if not wanted immediately.

Dehydration is carried out in various strengths of alcohol as usual, cleared in benzol, and then in pure benzyl benzoate, after which they are remarkably clear if illuminated with transmitted light. In direct light they exhibit a bluish opalescent sheen. This sheen, if objected to, can be removed by adding a very small quantity of oil of wintergreen to the benzol benzoate. For storing in benzol benzoate, add a small quantity of Beechwood creosote. Embryos done by Reagan's method are very beautiful.

810. Staining Embryological Sections.* Some years ago the routine methods for embryological study were borax carmine and picro-indigo-carmine, and a hæmatoxylin and eosin. We have yet to see preparations which surpass for beauty, really good borax carmine and picro-indigo-carmine sections. It is certainly true that some of the more recent polychromatic, many process staining methods are much inferior to the carmine ones. Nevertheless there has been a steady trend away from these older techniques towards the use of certain methods which give more information about the histology and cytology of development. It is quite possible nowadays to combine good embryological results with a satisfactory cytological and histological interpretation of the material being studied.

For the study of the histology of the gonads, as an example, the azan stain of Heidenhain, and Pasini, will be found excellent. Clearer cytological results will be obtained with Masson's iron

* §§ 810—815, suggested by Dr. J. P. Hill.

hæmatoxylin, fuchsin and anilin blue, while the safranin gentian violet and orange stain as used by de Winiwarter, is a classic method for studying the behaviour of the individual cells in developing organs. A more intensive attack on the cytological aspect can be made with the special methods described in the cytology sections (§§ 679, 738).

811.* **Heidenhain's Azan Stain.** This stain is good for gonads, and general histological methods. It resembles safranin somewhat, but gives a good cytological result, plus a fine histological demonstration of the various elements. Make a 2 per cent. solution of azocarmin in distilled water. Acidify strongly with glacial acetic acid (10 to 15 drops in a small slide staining jar). Stain sections in slide for one hour at 55° C. Rinse in water and differentiate in anilin alcohol (96 per cent. alcohol 100 c.c., anilin oil 0·1 c.c.); if differentiation is too slow, add 1 or 2 drops of anilin oil to the made-up solution till the correct strength is ascertained. Differentiate till cytoplasm is pale pink, and nuclei red and clear. Examine from time to time, first transferring slide to acid alcohol (96 per cent. alcohol, plus a few drops of acetic acid). Rinse in 96 per cent., plus a few drops glacial acetic. Transfer to 5 per cent. phosphotungstic acid until connective tissue is completely decolourised (about two hours). Rinse quickly in water and transfer to :—

Anilin blue (water sol. Grübler, etc.)	0·5 grm.
Orange G	2 grm.
Acetic acid	8 c.c.
Distilled water	100 c.c.

Dilute with from one to two times its bulk distilled water.

Stain one-half to three hours, examining from time to time under microscope. Wash in water, dehydrate in absolute alcohol, xylol, balsam.

812. Pasini's Method. Fix in alcohol, formalin, sublimate Zenker, etc. Prepare sections and stain in Erhlich or Delafield to show nuclei, differentiating well. Place sections from water into 2 per cent. phosphotungstic acid for ten minutes. Wash quickly in aq. dest., and stain for from fifteen to twenty minutes in the following mixture. Water-blue-orcein (Unna) 30 c.c., 2 per cent. Eosin B.A. (Grübler) in 50 per cent. alcohol, 30 c.c., saturated aqueous solution of säurefuchsin 4 c.c., neutral glycerin 15 c.c.

(The Unna's stain is made up as follows: (A) Wasserblau 1 grm. in 100 c.c. aq. dest., (B) orcein 1 grm. in 50 c.c. absolute alcohol, plus 5 c.c. acetic acid and 20 c.c. glycerin. Mix A and B.)

After staining in the water-blue-orcein acid fuchsin mixture,

* Refer to glands, §§ 889 to 909.

wash in distilled water slightly, dip into 70 per cent. alcohol, and differentiate in 90 per cent. alcohol.

At this stage the original method recommends downgrading to 50 per cent. alcohol and dipping into 2 per cent. phosphotungstic acid in water, and then upgrading to absolute, xylol and balsam. This stage may be omitted if it is found to extract the stain too much. Collagen fibres blue, protoplasm clear blue, keratohyalin red, keratin yellow red. This stain is excellent for demonstrating the structure of glands and genital organs. Unna's stain may be used alone, staining for twelve hours.

813. P. Masson's Iron Hæmatoxylin, Acid Fuchsin and Anilin Blue (*Traité de Pathologie Médicale*, Maloine, Paris, 1923). Prepare the following solutions: (A) Acid fuchsin, 1 grm., glacial acetic, 1 c.c., distilled water, 200 c.c. (B) Phosphomolybdic acid, 1 grm., distilled water, 100 c.c. (C) Anilin blue to saturation in 3 per cent. acetic acid in water, 100 c.c.

The anilin blue solution is prepared by boiling 100 c.c. of distilled water in a flask, adding 2 to 3 grm. of anilin blue, and then taking away the Bunsen. Two or three c.c. of acetic acid are added and the flask is plugged with cotton-wool and allowed to cool, and then the contents filtered.

Sections are first stained in iron-alum hæmatoxylin. The subsequent operations will take out some of the stain and the correct degree of under differentiation of the hæmatoxylin must be ascertained experimentally. Rinse in water and transfer to solution A for five minutes; rinse again and transfer to solution B for five minutes. Take out the slides and without rinsing, drop on 5 or 6 drops of solution C. Agitate the slide for a few moments till the blue mixes; time from two to five minutes to be ascertained experimentally. Rinse in aq. dest., place in 1 per cent. acetic acid for five minutes to one half-hour to wash off excess phosphomolybdic. Transfer slides to absolute alcohol with 1 per cent. acetic acid, thirty seconds—then pure absolute alcohol, and Canada balsam to which some salicylic acid has been added.

Results are chromatin black, cytoplasm and fibres red in various shades, collagen and mucus blue.

814. H. de Winiwarter's Safranin—Gentian Violet and Orange Triple Stain (*Arch. de Biol.*, xxxiii, 1923). **Fixation.** Flemming's fluid (twenty-four hours), or some one of the heavier osmicated chrome fixatives. De Winiwarter uses Meves' fluid (§ 47), and often adds urea according to E. Allen's procedure (§ 115). Staining is carried out on sections mounted on slides. The safranin must be a good one, bright and actively staining, without which the whole process will fail.

If the tissue has not been fixed in osmic fixatives, or has soaked for a long time in alcohol, the faculty for taking the triple stain

may be restored by treating the sections on the slide for twenty-four hours with strong Flemming. From 60 per cent. alcohol stain in a $\frac{1}{4}$ per cent. to 1 per cent. solution in 50 per cent. alcohol for twenty-four hours. Wash in several changes of distilled water, and then place in 1 per cent. aqueous gentian violet for twenty-four hours. Wash in aq. dest. Stain in aqueous solution of orange G for from some seconds to one minute. The orange G solution should be from 1 in 500 to 1 in 1000 in aq. dest. strength, varying according to the object, embryonic material needing the stronger solution. Finally treat sections with acidulated alcohol for a few seconds : on the first cloud of violet appearing, transfer to absolute alcohol. Transfer to oil of cloves containing a little absolute alcohol. Control differentiation under microscope. Then treat with pure oil, drain off, finally wash with xylol and mount in balsam.

815. Held's Molybdic Acid Hæmatoxylin. To a 1 per cent. solution of hæmatoxylin in 70 per cent. alcohol, add sufficient molybdic acid to form a layer on the bottom of the bottle. Shake frequently. After fourteen days, there is noticeable alteration in colour to deep blue-black. Solutions from months to one or two years' old stain best. Pour off the stain from the deposit, and just before use add some drops to some distilled water to make a semi-transparent solution. Stain at 50° C. or in cold up to twelve hours or more. Examination of the sections will reveal whether the stain is sufficient. Wash in water, and upgrade.

Alternatively, the sections may be mordanted in iron alum.

Overstained sections may be differentiated in 5 per cent. iron alum. Such fixatives as Zenker are suitable for this stain, but it may be used with almost any material. It is particularly good for nerve tissues during development.

For areas of osteoblastic activity, see § 921, and cartilaginous skeletons, § 923.

See also R. S. CUNNINGHAM (*Contrib. Carng. Inst. Wash.*, 1916, No. 12); L. H. WEED (*ibid.*, No. 14, 1917); F. SABIN (*Johns Hopkins Hosp. Report Monographs*, N.S., No. 5, Baltimore, 1913); *Contrib. to Embryol. Carneg. Inst. Wash.*, No. 7, 1915); P. G. SHIPLEY and C. C. MACKLIN (*Anat. Record*, x, 1915–16).

AVES

816. Superficial Examination. Instructions on this head are given in FOSTER and BALFOUR'S *Elements of Embryology*. The following is of more recent publication.

If it be desired to observe a living embryo by transmitted light, the egg should be opened under salt solution, as described below. A little of the white is then removed through the window, the egg is lifted out of the liquid, and a ring of gummed paper is

placed on the yolk so as to surround the embryonic area. As soon as the paper adheres to the vitelline membrane, which will be in a few minutes, a circular incision is made in the blastoderm outside the paper ring. The egg is put back into the salt solution, and the paper ring removed, carrying with it the vitelline membrane and the blastoderm, which may then be brought into a watch-glass or on to a slide and examined under the microscope (DUVAL).

Gerlach's Window Method (*Nature*, 1886, p. 497). Remove with scissors the shell from the small end of the egg; take out a little white by means of a pipette; the blastoderm will become placed underneath the window just made, and the white that has been taken out may be replaced on it. Paint the margins of the window with gum mucilage, and build up on the gum a little circular wall of cotton-wool; place on it a small watch-glass (or circular cover-glass), and ring it with gum. When the gum is dry the cover is further fixed in its place by means of collodion and amber varnish, and the egg is put back in its normal position in the incubator. The progress of the development may be followed up to the fifth day through the window.

A description of further developments of this method, with figures of special apparatus, will be found in *Anat. Anz.*, ii, 1887, pp. 583–609.

See also PATON, *Journ. Exper. Zool.*, xi, 1911, p. 469 (cultivation of the embryo *in vitro*).

817. Preparation. During the first twenty-four hours of incubation, it is extremely difficult to separate the blastoderm from the yolk, and they should be fixed and hardened together. ANDREWS (*Zeit. wiss. Mik.*, xxi, 1904, p. 177) separates the blastoderm at this stage by injecting picro-sulphuric acid (not any rapidly acting fixative) firstly, between the blastoderm and the vitelline membrane, so as to separate the two above, and then between the blastoderm and the yolk, so as to free the blastoderm below and float it up. This done, the membrane may be incised and the blastoderm removed. The injection is best done with a pipette having a fine point bent upwards.

In later stages, when the embryo is conspicuous, the blastoderm can easily be separated from the yolk, which is very advantageous. To open the egg, lay it on its side and break the shell at the broad end by means of a sharp rap; then carefully remove the shell bit by bit by breaking it away with forceps, working away from the broad end until the blastoderm is exposed. The egg should be opened in salt solution, then lifted up a little, so as to have the blastoderm above the surface of the liquid; the blastoderm is then treated with some fixing solution dropped on it from a pipette (1 per cent. solution of osmic acid, or Ranvier and Vignal's osmic acid and alcohol mixture, iodised serum, solution of Kleinenberg, 10 per cent. nitric acid, etc.). By keeping the upper end of the pipette closed, and the lower end in contact with the liquid on the blastoderm, the blastoderm may be kept well immersed for a few minutes, and should then be found to be

sufficiently fixed to be excised. (Of course, if you prefer it, you can open the egg in a bath of any fixing liquid [10 per cent. nitric acid being convenient for this purpose] of such a depth as to cover the yolk ; and having exposed the blastoderm, leave it till fixed [fifteen to twenty minutes] ; but we think the procedure above described will generally be found more convenient.)

The egg is put back into the salt solution, and a circular incision made round the embryonic area. The blastoderm may then be floated out and got into a watch-glass, in which it may be examined or may be brought into a hardening liquid.

Before putting it into the hardening fluid, the portion of vitelline membrane that covers the blastoderm should be removed with forceps and shaking.

Fixation in 10 per cent. nitric acid has the advantage of greatly facilitating the separation of the blastoderm. The acid snould be allowed to act for ten minutes, after which it is well to bring the preparation into 2 per cent. solution of alum (*cf.* HOFMANN, *Zeit. wiss. Mik.*, x, 1893, p. 485). MITROPHANOW (*Anat. Hefte*, xii, 1899, p. 200) fixes with nitric acid of 3 per cent. ; SUSCHKIN (*Nouv. Mém. Soc. Nat. Moscow*, xvi, 1899, p. 34) with sublimate ; FISCHEL (*Morph. Jahrb.*, xxiv, 1896, p. 371) with Rabl's platino-sublimate, § 81 (embryos of the duck) ; PATTERSON (*Biol. Bull. Wood's Hole*, xiii, 1907, p. 252) with picro-sulphuric acid containing 8 per cent. of acetic acid, for an hour (ova of *Columba*) ; HOSKINS (*Kansas Univ. Sci. Bull.*, iv, 1907, p. 176), after removing shell, for five to fifteen minutes in a mixture of 3 parts of 10 per cent. formol with 1 of 10 per cent. nitric acid, and then excises the embryo.

In order to counteract the turning up of the edges of the blastoderm that generally happens during the process of hardening, it is well to get the blastoderm spread out on the *convex* surface of a watch-glass, and leave it so during the hardening.

For hardening HENNEGUY prefers the osmic acid and alcohol mixture of Ranvier and Vignal, or Flemming's mixture followed by successive alcohols.

Stain and imbed by the usual methods.

Up to about the fiftieth hour embryos may be mounted entire in glycerin or balsam.

818. M. DUVAL's **Orientation Method** (*Ann. Sc. Nat.*, 1884, p. 3). In the early stages of the development of the ova of Aves, before the appearance of the primitive streak, it is difficult to obtain a correct orientation of the hardened cicatricula, so as to be able to make sections in any desired direction. DUVAL, starting from the fact that during incubation the embryo is almost always found to be lying on the yolk in such a position that the big end of the egg is to the left and the little end to the right of it, marks the position of the blastoderm in the following way.

With a strip of paper 5 mm. wide and 50 mm. long you construct a sort of triangular bottomless box. You lay this on the yolk, enclosing the cicatricula in such a position that the base of the triangle corresponds to what will be the anterior region of the embryo, and its apex to the posterior region; that is to say, if the big end of the egg is to your left, the apex of the triangle will point towards you. You now, by means of a pipette, fill the paper triangle with 0·3 per cent. solution of osmic acid. As soon as the preparation begins to darken you put the whole egg into weak chromic acid, remove the white, and put the rest into clean chromic acid solution for several days. After hardening you will find on the surface of the yolk a black triangular area, which encloses the cicatricula and marks its position; you cut out this area with scissors and a scalpel, and complete the hardening with chromic acid and alcohol.

See also the method of Hirota, *Journ. Roy. Mic. Soc.*, 1895, p. 118.

819. Kionka's **Orientation Method** (*Anat. Hefte*, 1 Abth., iii, 1894, p. 414). Open the egg under salt solution, free it from the shell and albumen, and mark the poles by sticking into it, at about a centimetre from the blastoderm, two hedgehog spines, the one at the obtuse end being marked with a red thread. Put the whole for ten minutes into water at 90° C., then bring into 70 per cent. alcohol, and after twenty-four hours cut out the blastoderm and a little yolk round it in the shape of an isosceles triangle, whose base marks the anterior end of the blastoderm. Paraffin sections stained with borax-carmine, washed out with acid alcohol containing 1 drop of concentration solution of Orange G for each 5 c.c., which stains the yolk.

820. Vialleton's **Method** (*Anat. Anz.*, vii, 1892, p. 624). Egg opened in salt solution, blastoderm excised and removed to a glass plate, then treated with 1 per cent. nitrate of silver solution, washed with water, and put into 70 per cent. alcohol, for six to twelve hours in the dark. Borax-carmine, alcohol, damar.

821. Chick and Reptile Blastoderms. Gerhardt (*Anat. Anz.*, xx) uses :—

Chromic acid 1 per cent. . . .	150 c.c.
Sat. corr. subl.	150 ,,
Aq. dest.	135 ,,
Acetic acid	15 ,,
Formalin	150 ,,

Leave in twenty-four hours. Wash twenty-four hours in running water, upgrade from 70 per cent. alcohol, 90 per cent. with iodine, pure 90 per cent., etc. Recommended by Prof. J. P. Hill.

REPTILIA

822. General Directions. The methods described above for birds are applicable to reptiles. During the early stages the blastoderm should be hardened *in situ* on the yolk; later the embryo can be isolated, and treated separately.

Böhm and Oppel (*Taschenbuch*, 1900, p. 186) remove the shell under salt solution, fix in sublimate with 20 per cent. acetic acid, or in Lo Bianco's chromo-sublimate (§ 77), then remove the blastoderm and bring it into alcohol.

823. Special Cases. Mitsukuri (*Journ. Coll. Sc. Japan*, vi, 1894, p. 229) fixes embryos of tortoises chiefly with picro-sulphuric acid. To study the blastoderm he removes the whole of the shell and as much as possible of the albumen, marks the place where the blastoderm lies with a hair, brings the whole, with the blastoderm uppermost, into the fixative, and after a few hours cuts out the blastoderm and further hardens it by itself. Young embryos generally adhere to the shell and can, therefore, be fixed in a piece of it made to serve as a watch-glass, then after half an hour can be removed from it and further hardened alone. If the embryonal membranes have been formed, the shell may be scraped away at some spot and there treated with picro-sulphuric acid until a small hole is formed; then by working away from this spot, by means of scraping and dropping acid on to it, the whole of the shell may be removed.

Will (*Zool. Jahrb., Abth. Morph.*, vi, 1892, p. 8) opens ova of *Platydactylus* in the fixative (chiefly chromic acid, or chromo-aceto-osmic acid with very little osmic acid) and hardens the embryos on the yolk; so also for *Cistudo* and *Lacerta* (1893 and 1895). Mehnert (*Anat. Anz.*, xi, 1895, p. 257) does not approve of these methods; for his own see *Morph. Arb. Schwalbe*, i, 1891, p. 370.

Gerhardt (*Anat. Anz.*, xx, 1901, p. 244) fixes ova of *Tropidonotus* for twenty-four hours in Nowak's mixture, § 117.

Ballowitz (*Entwickl. d. Kreuzotter*, 1903, p. 19) first fixes segments of the uterus, each containing an ovum, for one or two hours, then tears them open with forceps, isolates the ova, and puts them into fresh fixative, and thence into alcohol of 40 per cent.

Nicolas (*Arch. Anat. Mic.*, 1900, p. 457) finds the best fixative for ova of the slow-worm, as for other large ova, is Bouin's picro-formol (§ 115).

See also Perényi, § 45, and *Zool. Anz.*, 1888, pp. 139 and 196, and other methods in *early editions*.

AMPHIBIA

824. Preliminary. In order to prepare ova for section-cutting, it is essential to begin by removing their thick coats of albumen.

This may be done by putting them for two or three days into 1 per cent. solution of chromic acid, and shaking well; but ova thus treated are very brittle, and do not afford good sections. A better method is that described by WHITMAN (*Amer. Natural.*, xxii, 1888, p. 857), and by BLOCHMANN (*Zool. Anz.*, 1889, p. 269). WHITMAN puts the fixed eggs into a 10 per cent. solution of sodium hypochlorite diluted with 5 to 6 volumes of water, and leaves them there till they can be shaken free, which happens (for *Necturus*) in a few minutes. BLOCHMANN takes *eau de Javelle* (potassium hypochlorite), and dilutes it with 3 to 4 volumes of water, and agitates the eggs, previously fixed with solution of Flemming, for fifteen to thirty minutes in it.

LEBRUN (*La Cellule*, xix, 1902, p. 316) advises fixing ova of Anura for not less than one and a half hours in liquid of Gilson, § 74. The outer envelopes are then hard, and may be easily incised and the ovum extracted by pressing on the pole opposite to the incision. The operation should *not* be delayed until after hardening in alcohol. Similarly (*ibid.*, xix, 1902, p. 12) for Urodela.

GUYER (*Amer. Nat.*, xli, 1907, p. 400) finds it suffice to roll the ova (either fresh or fixed, but before bringing into alcohol) on blotting paper.

825. Imbedding. A great difficulty with the ova of Amphibia lies in their becoming extremely brittle on imbedding in paraffin. CARNOY and LEBRUN (*La Cellule*, xii, 1897, p. 212) fix ovaries or ovarian ova for fifteen minutes to three-quarters of an hour (but see last §) in Gilson's mercuro-nitric fluid, § 74, and preserve them in 80 per cent. alcohol. To imbed, they are brought for a quarter of an hour into 95 per cent. alcohol, five minutes in absolute alcohol, then into a mixture of alcohol and chloroform in equal parts, and as soon as they sink in that they are put into pure chloroform. Paraffin is added to the chloroform, enough to about double the volume of the whole, and the whole is put for about three hours into a stove at 35° C. Lastly, the ova are put *for not more than five minutes* into a bath of pure paraffin at 52° C.

LEBRUN (*ibid.*, xix, 1902, p. 317) explains that it is important not to dehydrate completely with absolute alcohol; the ova should be left in alcohol of 96 per cent. until chloroform can be added without the mixture becoming turbid, and a second bath of clean paraffin should be added.

See also MORGAN, *Devel. of the Frog's Egg*, New York, 1897, p. 171.

826. Siredon. The ova are easier to prepare than those of the Anura, because the yolk is separated from the albuminous layer by a wide space filled with a liquid that is not coagulated by reagents. Put the eggs for a few hours into picro-sulphuric acid,

then pierce the inner chorion with fine scissors or needles, and gently press out the ovum. Harden in alcohol.

Fick (*Zeit. wiss. Zool.*, lvi, 1893, p. 529) uses a mixture of 250 parts of 1 per cent. chromic acid, 1 of acetic acid, and 750 of water.

827. Triton (Scott and Osborn, *Quart. Journ. Mic. Soc.*, 1879, p. 449). The albumen is here present in the form of several concentric coats, which are very delicate. Incise each of them separately with fine scissors, turn out the ovum, and fix it in solution of Kleinenberg.

Hertwig (*Jen. Zeit. Naturw.*, 1881–2, p. 291) puts the eggs into a mixture of equal parts of 2 per cent. acetic acid and 0·5 per cent. chromic acid. After ten hours he incises the membranes, opening one end of the inner chorion, and turns out the embryos and brings them into successive alcohols.

Michaelis (*Arch. mik. Anat.*, xlviii, 1896, p. 528) fixes ova, with their envelopes, in a mixture of concentrated sublimate solution and concentrated picric acid, 20 parts each, glacial acetic acid 1, and water 40, but removes the envelopes before bringing into alcohol.

828. Salamandra (Rabl, *Morphol. Jahrb.*, xii, 2, 1886, p. 252). For his more recent methods see § 834.

Grönross (*Anat. Anz.*, xiv, 1898, p. 461) fixes the ova with a mixture of 50 parts each of saturated sublimate and 0·5 per cent. chromic acid with 1 part of acetic acid.

829. Rana. Gatenby has found that the following mixture often gives very good results for the *eggs* (not embryos) of Rana temporaria ; it dissolves away the albumen coat, preserves yolk and mitochondria, and leaves the eggs soft enough to cut in paraffin with a rotary microtome :—

Bichromate of potash of 2 per cent. .	100 c.c.
Chromic acid of 1 per cent. . . .	100 ,,
Nitric acid	6 ,,

Use at least 40 c.c. to twenty or thirty eggs for fifteen to twenty-four hours. Slightly shake, and the albumen coats fall off if not already dissolved. Wash out for about one hour in running water and then upgrade from 70 per cent. alcohol (one half-hour), 90 per cent. (one hour), to absolute alcohol, two changes of one hour each. Clear in benzol for fifteen minutes. Add chips of wax and place in thermostat for half an hour. Transfer to pure wax for one-half (to three-quarters) of an hour. Avoid unnecessary heat.

The eggs thus treated can often be cut 6 μ on a rotary microtome provided with a *sharp* knife. The method is indicated where large numbers of stages of the early development of the frog are required for junior class purposes. The main objection to the method is that the chromic acid attacks pigment. Fertilisation and segmentation stages and general cytology are often extremely good ; stain in any way.

O. Hertwig (*Jen. Zeit. Naturw.*, xvi, 1883, p. 249).—The ova are thrown into nearly boiling water (90° to 96° C.) for five or ten minutes.

The albuminous envelope of the ovum is then cut open, and the ovum extracted under water. The ova are then brought into 0·5 per cent. chromic acid for not more than twelve hours, or into alcohol of 70, 80, and 90 per cent. Chromic acid makes ova brittle and attacks the pigment, whilst alcohol preserves it, which is frequently important for the study of the germinal layers.

MORGAN (*Amer. Nat.*, xxv, 1891, p. 759, and *Devel. of the Frog's Egg*, 1897, p. 171) has the following. During the periods in which it is difficult or impossible to remove the inner jelly-membrane the eggs can be freed as follows: Each egg is cut out with scissors from the general jelly-mass, and put for from one to twelve hours into saturated solution of picric acid in 70 per cent. alcohol containing 2 per cent. of sulphuric acid. Wash in several changes of alcohol of 70 per cent. About the second day in this the inner membrane begins to swell, and on the third or fourth day may be pierced by a needle, and the egg removed and placed in 80 per cent. alcohol. See also WHITMAN, *Meth. of Research*, p. 156.

SCHULTZE (*Arch. mik. Anat.*, lv, 1899, p. 174) removes with scissors the outer layers of albumen, and puts the ova for five minutes in 2 per cent. formol warmed to 75° or 80° C. The membrane left on the ova then rises up sufficiently to allow the ova to be got out with needles.

See also BORN (*ibid.*, xliii, 1894, p. 1).

H. D. KING (*Journ. Morph.*, xvii, 1901, p. 295, and xix, 1908, p. 370) fixes (for a few minutes) the spawn (of *Bufo*) in sublimate (saturated with 5 per cent. of acetic acid), or in Flemming Zenker, or Hermann, brings into alcohol, first of 50 and then 80 per cent., and removes the jelly after a few days.

BLES (*Trans. Roy. Soc. Edinburgh*, xli, 1905, p. 792) takes for ova, formol of 10 per cent., but for embryos and larvæ the mixture given § 114.

BOUIN takes for larvæ of *Rana* the formol-sublimate mixture § 117.

830. Sulphate of Copper Liquid (FOL, *Lehrbuch*, p. 106, after REMAK and GOETTE); for hardening ova of Amphibia:

2 per cent. solution of sulphate of copper .	50 c.c.
Alcohol of 25 per cent.	50 „
Rectified wood vinegar.	35 drops.

PISCES

831. Teleostea in General. The ova of many of the bony fishes can be studied by transmitted light in the living state; but those of the Salmonidæ must be hardened and removed from their envelopes for the study of the external forms of the embryo.

To this end they may be put for a few minutes into water containing 1 to 2 per cent. of acetic acid, and thence into 1 per cent. chromic acid. After three days the capsule of the ovum may be opened at the side opposite to the embryo, and be removed with fine forceps. The ovum is put for twenty-four hours into distilled water, and then into successive alcohols. Embryos thus prepared show no deformation, but the vitellus rapidly becomes excessively hard and brittle, so as greatly to interfere with section-cutting.

The following processes give good results as regards section-cutting.

Put the ova for a few minutes into 1 per cent. osmic acid; as soon as they have taken on a light brown colour bring them into Müller's solution. Open them therein with fine scissors—the vitellus, which immediately coagulates on contact with air, dissolves, on the contrary, in Müller's solution—and the germ and cortical layer can be extracted from the capsule of the ovum. They should be left in clean Müller's solution for a few days, then washed with water for twenty-four hours, and brought through successive alcohols.

Another method (Henneguy) is as follows: The ova are fixed in solution of Kleinenberg containing 10 per cent. of acetic acid. After ten minutes they are opened in water containing 10 per cent. of acetic acid, which dissolves the vitellus. The embryos are put for a few hours into pure solution of Kleinenberg, and are then brought through alcohol of gradually increasing strength.

Child (quoted from Sumner, *Mem. New York Acad. Sci.*, ii, 1900, p. 78) fixes for about a minute in sublimate with 10 per cent. of acetic acid, and brings into formalin of 10 per cent., which is said to give a good fixation of the embryo without the yolk becoming hard.

832. Kollmann's **Fixative** (Kollmann, *Arch. Anat. Phys.*, 1885, p. 296).

Bichromate of potash	5 gm.
Chromic acid	2 ,,
Concentrated nitric acid	2 ,,
Aq. dest.	100 c.c.

For ova of Teleostea. Fix for twelve hours, wash with water for twelve hours, then remove the chorion, and put the ova into 70 per cent. alcohol.

833. Rabl's **Method**, see § 834; for Kowalewsky's see *Zeit. wiss. Zool.*, xliii, 1886, p. 434, or *Third Edition*.

834. Salmonidæ. Henneguy's methods have been given, § 831.

Kopsch (*Arch. mik. Anat.*, li, 1897, p. 184), on the suggestion of Virchow, fixes embryos for five or ten minutes in a mixture of 1 part of chromic acid to 50 of glacial acetic acid and 450 of water, then removes into chromic acid of 1 : 500, and as soon as may be removes the capsule and yolk *under salt solution* and completes the hardening in the chromic acid or the saturated sublimate solution.

Similarly, Behrens (*Anat. Hefte*, x, 1898, p. 233). He opens the ova in the salt solution from the *antipolar* side, and frees the embryo from the yolk that remains by blowing the latter away with a fine-pointed glass tube.

Similarly also Sobotta (*ibid.*, 1902, p. 579).

GUDGER (*Proc. U.S. Nation, Mus.*, xxix, 1906, p. 448) fixes blastoderms in fresh liquid of Perényi, which does not make the yolk too hard; later stages in WORCESTER's liquid (9 parts of saturated solution of sublimate in formol of 10 per cent. and 1 part of acetic acid), for half an hour to an hour, and brings gradually into alcohol of 70 per cent.

BOUIN (*C. R. Soc. Biol.*, lv, 1903, p. 1691) fixes for thirty-six to forty-eight hours in picro-formol.

RABL-RÜCKHARD's **Method** (*Arch. Anat. Entw.*, 1882, p. 118). Fix in 10 per cent. nitric acid for fifteen minutes. Remove the membranes to avoid deformation of the embryos, and put the ova back into the acid for an hour. Wash out in 1 to 2 per cent. solution of alum for an hour and harden in alcohol.

Modification of this method by GORONOWITSCH (see *Morph. Jahrb.*, x, 1884, p. 381).

835. Selachia. BEARD (*Anat. Anz.*, xviii, 1900, p. 556) has found that the best fixatives for embryos of *Raja* are Rabl's picro-platinic mixture, § 80, and sublimate.

Living embryos can be observed by scraping the shell thin with a knife (KASTSCHENKO, *Anat. Anz.*, iii, 1888, p. 445, and HIS, *Arch. Anat. Phys., Anat. Abth.*, 1897, p. 3). See also BRAUS, *Morph. Jahrb.*, xxxv, 1906, p. 250.

836. Amphioxus. SOBOTTA (*Arch. mik. Anat.*, l. 1897, p. 20) fixes for twenty-four hours in liquid of Flemming; HATSCHEK (*Arb. Zool. Inst. Wien*, iv, 1881) in picro-sulphuric acid. Impregnation takes place in the evening, and segmentation is completed during the night.

LEGROS (*Grundzüge*, LEE and MAYER, 1910, p. 288) fixes ova and embryos in equal parts of formol and Flemming. Sublimate is not good; Rabl's mixtures are better. Larvæ and young animals ought first to be anæsthetised with cocaine in sea-water. After fixation they should remain only for as short a time as possible in alcohol.

CERFONTAINE (*Arch. Biol.*, xxii, 1906, p. 287) fixes with Flemming or Hermann. For study of ova *in toto* he orients them on a slide in clove-oil collodion which he sets with chloroform, and adds balsam. For sectioning, he orients in the same way on a layer of paraffin spread on a cover-glass and imbeds the whole in paraffin.

837. Pelagic Fish Ova. WHITMAN (*Amer. Natural.*, xvii, 1883, pp. 1204–5; and *Methods of Research*, etc., p. 152). Fix by treatment first for five to ten minutes with a mixture of equal parts of sea-water and ½ per cent. osmic acid solution, and then for one or two days with a solution (due to Eisig) of equal parts of 0·25 per cent. platinum chloride and 1 per cent. chromic acid. Prick the membrane before transferring to alcohol. See also AGASSIZ and WHITMAN, in *Proc. Amer. Acad. Arts and Sciences*, xx, 1884; and COLLINGE, *Ann. and Mag. Nat. Hist.*, x, 1892, p. 228.

RAFFAELE (*Mitth. Zool. Stat. Neapel*, xii, 1895, p. 169) fixes chiefly with liquid of Hermann (1 to 2 days), or with a mixture of Mingazzini (absolute alcohol 1, acetic acid 1, saturated sublimate solution in water 2).

HEINKE and EHRENBAUM (*Wiss. Meeresunt. Komm. Wiss. Unt. D. Meere*, iii, Heligoland, 1900, pp. 205 and 213) prefer formol with 39 volumes of sea-water.

TUNICATA

838. Ova. DAVIDOFF (*Mitth. Zool. Stat. Neapel*, ix, 1, 1889, p. 118) fixes the ova of *Distaplia* with a mixture of 3 parts of saturated solution of corrosive sublimate and 1 of glacial acetic acid for from half an hour to an hour; or with a mixture of 3 parts of saturated solution of picric acid and 1 of glacial acetic acid for three to four hours; then 70 per cent. alcohol.

CASTLE (*Bull. Mus. Harvard Coll.*, xxvii, 1896, p. 213) advises for ova of *Ciona* liquid of Perényi for twenty minutes, followed by 70 per cent. alcohol for twenty-four hours, and for the larvæ picro-nitric acid.

839. Test-Cells of Ascidians (MORGAN, *Journ. of Morphol.*, iv, 1890, p. 195).—Tease fresh ovaries in very weak osmic acid, wash in distilled water, treat for half an hour with 1 per cent. silver nitrate, wash for half an hour in 2 per cent. acetic acid and reduce in sunlight. Imbed in paraffin. By this process the *limits* of the follicle cells are demonstrated.

840. Buds. PIZON (*Ann. Sc. Nat.*, xix, 1893, p. 5) studies the gemmation of the composite Ascidians either on entire corms, which he first bleaches with peroxide of hydrogen and then stains, or by making sections, after anæsthetising the colonies with cocaine of 1 : 1000, fixing in glacial acetic acid or picro-sulphuric or liquid of Flemming, and staining *in toto* with borax carmine or alum carmine, or with a strong solution of methylen blue in alcohol of 90 or 100 per cent. (after BERNARD, *ibid.*, ix, 1890, p. 97).

RITTER (*Journ. of Morph.*, xii, 1896, p. 150) recommends for fixing *Perophora* and *Goodsiria* picro-sulphuric acid.

BRYOZOA

841. Statoblasts. BRAEM (*Bibl. Zool. Chun and Leuckart*, 6 Heft, 1890, p. 95) fixes statoblasts of *Cristatella* with hot concentrated solution of sublimate for ten minutes, brings them into water and there incises them with a razor, and after half an hour passes them gradually into alcohol. He stains with picro-carmine.

MOLLUSCA

842. Cephalopoda (USSOW, *Arch. de Biol.*, ii, 1881, p. 582). Segmenting ova are placed in 2 per cent. solution of chromic

acid for two minutes, and then in distilled water, to which a little acetic acid (1 drop to a watch-glassful) has been added, for two minutes. If an incision be now made into the egg-membrane, the yolk flows away and the blastoderm remains; if any yolk still clings to it, it may be removed by pouring away the water and adding more.

WATASÉ (*Journ. of Morph.*, iv, 1891, p. 249) kills the ova in the macerating mixture of the Hertwigs (§ 568), and as soon as the blastoderm turns white and opaque removes it under dilute glycerin. Treatment with liquid of Perényi is recommended for surface views.

VIALLETON (*Ann. Sc. Nat.*, vi, 1887, p. 168) brings ovarian ova of *Sepia* into a freshly prepared mixture of picro-sulphuric acid and 2 per cent. solution of bichromate of potash in equal parts, and after one or two minutes incises them in the equator, fixes for an hour and a half in picro-sulphuric acid the halves that contain the formative vitellus, separates this from the nutritive vitellus with a spatula, spreads it out, and hardens it in alcohol of 70 to 90 per cent. He fixes entire ova in liquid of Flemming or osmic acid.

KORSCHELT (*Festschrift Leuckart*, Leipzig, 1892, p. 348) fixes advanced embryos of *Loligo* in liquid of Flemming, sublimate, picro-sulphuric acid, or 0·2 per cent. chromic acid. This last is specially good for young embryos if it is washed out with many changes of picric acid.

FAUSSEK (*Mitth. Zool. Stat. Neapel*, xiv, 1900, p. 83) recommends picro-nitric acid. Fix in this, harden in alcohol, bring the ova, still in their albumen, into hæmalum, stain for twenty-four hours, wash in 1 per cent. alum solution for twenty-four hours, when the albumen will be found softened so that the ova can easily be extracted.

843. Gastropoda (HENNEGUY). Ova of *Helix* may be fixed for from four to six hours in Mayer's picro-nitric acid. The carbonate of lime that encrusts the external membrane is thus dissolved, and the albuminous coat of the egg is coagulated. The egg is opened with needles, the albumen comes away in bits, and the embryo can be removed.

HENCHMAN (*Bull. Mus. Comp. Zool., Harvard*, xx, 1890, p. 171) fixes ova of *Limax* with 0·33 per cent. chromic acid, or with liquid of Perényi. It is best to remove only the outer envelope before putting into the chromic acid, the inner membrane being removed after two or three minutes therein. Where Perényi is used the membranes must be removed first, as the albumen will else coagulate in such a way as to prevent the removal of the embryos.

MEISENHEIMER (*Zeit. wiss. Zool.*, lxii, 1896, p. 417) dissects out the embryos of *Limax* and fixes them with picro-sulphuric

acid or concentrated sublimate. Advanced embryos are first got into extension by means of 2 per cent. cocaine, or are rapidly killed with hot sublimate.

SCHMIDT (*Entw. Pulmonaten*, Dorpat, 1891, p. 4) fixes the ova *in toto* with concentrated sublimate, and dissects them out afterwards.

Similarly KOFOID (*Bull. Mus. Harvard Coll.*, xxvii, 1895, p. 35). Or, preferably, the ova are put into salt solution, the shell removed, the albumen removed with a pipette full of salt solution, which dissolves it; the ova are then fixed for one minute in Fol's modification of liquid of Flemming, and brought direct into Orth's picro-lithium-carmine. See also LINVILLE, *ibid.*, 1900, p. 215, who adopts this method of shelling, but prefers fixing in acetic-acid sublimate, or liquid of Perényi.

HEYDER (*Zeit. wiss. Zool.*, xciii, 1909, p. 92), before imbedding embryos of *Arion* that have been fixed with sublimate, treats them for an hour or two with carbonate of soda of $\frac{1}{10}$ to $\frac{1}{15}$ per cent., which makes the stomach and intestine less brittle.

GATENBY (*Quart. Journ. Micr. Science*, 1919), for *Limnæa stagnalis* ova, used Flemming's strong fluid without acetic acid, Champy's fluid for two days to a week, and Kopsch's method.

HOLMES (*Journ. of Morph.*, 1900, p. 371) teases the egg-capsules of *Planorbis* in nitrate of silver of $\frac{3}{4}$ per cent., exposes to sunlight until the cell-limits come out, rinses with 0·2 per cent. hyposulphite of soda, puts for a few minutes into picric acid, and then through alcohol into balsam.

See also WASHBURN, *Amer. Anat.*, xxviii, 1894, p. 528 (liquid of Flemming, or 0·3 per cent. chromic acid, or 1 per cent. osmic acid, followed by liquid of Merkel).

CONKLIN (*Journ. of Morph.*, xiii, 1897, p. 7) fixes ova of *Crepidula* for fifteen to thirty minutes in picro-sulphuric acid, and stains with dilute acidified hæmatoxylin of Delafield.

KOSTANECKI and WIERZEJSKI (*Arch. mik. Anat.*, xlvii, 1896, p. 313) fix the spawn of *Physa fontinalis* either in $1\frac{1}{2}$ to 2 per cent. nitric acid, or in "sublimate and 3 per cent. nitric acid in the proportion of 2 : 1," and bring through successive alcohols. They imbed entire ova in paraffin, but isolated embryos in celloidin.

844. CHITON, see METCALF, *Stud. Biol. Lab. Johns Hopkins Univ.*, v, 1893, p. 251. (Ova with young embryos put for twenty to forty-five seconds into *eau de Labarraque*, then into water, in which the chorion swells and can easily be removed.)

845. Lamellibranchiata. STAUFFACHER (*Jena Zeit.*, xxviii, 1893, p. 196) fixes embryos of *Cyclas* in sublimate, stains with hæmalum, and cuts in paraffin.

LILLIE (*Journ. of Morph.*, x, 1895, p. 7) fixes ova of *Unio* for ten to twenty minutes in liquid of Perényi, and preserves them in 70 per cent. alcohol, or advanced embryos with liquid of Merkel

or sublimate, larvæ with 0·05 to 0·1 per cent. osmic acid, preserving them in glycerin. Glochidia may be cut with the shell in paraffin of 58° melting-point; they may be anæsthetised with chloral hydrate before fixing.

ARTHROPODA *

846. Fixation of Ova. In many cases the ova of Arthropods are best fixed by heat (§ 11). This may be followed either by alcohol or some watery hardening agent. If it be desired to avoid heating, picro-nitric acid may be tried.

Heavy yolked insect eggs are dealt with as follows by Miss E. H. Slifer and R. L. King (*Science*, 1933). This is a modification of A. Petrunkewitsch's cupric-phenol method, and is used by Miss Slifer and R. King especially for grasshopper (locust) eggs.

Fix in Carnoy-Lebrun. Wash in iodised alcohol, cut in half and store the micropyle halves in 75 per cent. alcohol. When needed, expose for twenty-four hours to 4 per cent. phenol in 80 per cent. alcohol, dehydrate in 95 per cent. alcohol, clear in carbol-xylol, infiltrate with paraffin, each one blocked with the cut end outwards. Trim until the yolk is just exposed and soak in water for twenty-four to forty-eight hours. This eliminates the necessity of moistening each section separately.

Another method is to fix in Bouin overnight. Transfer to the phenol-alcohol solution mentioned above for twenty-four hours. Imbed and section. Good for chromosomes.

847. Removal of Membranes. It may often be advisable not to attempt to remove them, but to soften them with *eau de Javelle* or *eau de Labarraque*. See § 586.

Morgan (*Amer. Natural.*, xxii, 1888, p. 357) recommends (for the ova of *Periplaneta*) *eau de Labarraque* diluted with 5 to 8 volumes of water, and slightly warmed. This will soften the chitin membranes sufficiently in thirty to sixty minutes, if employed before fixing. Fixed ova take longer. The fluid must, of course, not be allowed to penetrate into the interior of the ovum.

848. Henking's Methods (*Zeit. wiss. Mik.*, viii, 1891, p. 156). Henking generally kills ova by plunging them into hot water, or by pouring hot water on to them in a watch-glass, and then removing into 70 per cent. alcohol.

He thinks that *eau de Javelle* for softening membranes is best avoided. They should either be dissected away or left *in situ*, and cut with the rest of the egg, according to the nature of the case. To avoid brittleness of the yolk proceed as follows: After fixing and treating with alcohol, prick the chorion and stain with borax-carmine. Put the stained ova for twelve hours into a mixture containing 20 c.c. of 70 per cent. alcohol, 1 drop of

* See remarks in §§ 1182 *et seq.*

concentrated hydrochloric acid, and a knife pointful of pepsin (it is not necessary that all the pepsin should be dissolved). The ova may then be treated with alcohol, oil of bergamot, and paraffin, and (with some exceptions, amongst which is *Bombyx mori*) will be found to cut without crumbling.

849. Diptera (Henking, *Zeit. wiss. Zool.*, xlvi, 1888, p. 289). Ova still contained within the fly may be fixed by plunging the insect for some time into boiling water, then dissecting out and bringing them into 70 per cent. alcohol. Laid eggs may have boiling water poured over them, or be put into solution of Flemming in a test-tube which is plunged into boiling water until the eggs begin to darken (about a minute). Cold solution of Flemming easily causes a certain vacuolisation of the contents of the ova. Open the ova at the larger end, stain with borax-carmine for fifteen to thirty hours, and cut in paraffin.

Bruel (*Zool Jahrb., Abth. Morph.*, x, 1897, p. 569) fixes larvæ and pupæ in absolute alcohol heated to 70° to 75° C., and containing a "little" sublimate. See also Van Rees, *ibid.*, iii, 1888, p. 10.

Bengtsson (*Handl. Fysiogr. Sællsk Lund.*, viii, 1897) finds hot alcoholic solution of sublimate (Frenzel's, § 74) the best fixative for larvæ of *Phalacrocera*. He could not succeed in softening the chitin with *eau de Javelle*.

Pérez (*Arch. Zool. expér.*, (4), v, 1910, p. 11) fixes pupæ in Bouin's picro-formol, or Marchoux's mixture, for twenty-four hours.

850. Lepidoptera (Bobretzky, *Zeit. wiss. Zool.*, 1879, p. 198). Ova are slightly warmed in water and put for sixteen to twenty hours in 0·5 per cent. chromic acid. The membranes can then be removed.

851. Hymenoptera. Carriére and Bürger (*Nova Acta Acad. Leop. Car.*, lxix, 1897, p. 273) kill ova of *Chalicodoma* by warming in water to 60° C., and fix in aqueous picric acid, or alcohol of 70 per cent.

Petrunkewitsch (*Zool. Jahrb., Abth. Morph.*, xiv, 1901, p. 576) fixes for twenty-four hours in his sublimate mixture, and passes into alcohol of 70 per cent. with iodine.

852. Orthoptera (Patten, *Quart. Journ. Mic. Sci.*, 1884, p. 549). The ova or larvæ (of Blattidæ) are placed in cold water, which is gradually raised to 80° C. You leave off heating as soon as the ova have become hard and white. Pass very gradually through successive alcohols, beginning with 20 per cent.

Wheeler (*Journ. of Morph.*, iii, 1889, p. 292) dissects out ovarian ova in salt solution and fixes in liquid of Perényi (fifteen minutes), then treats with alcohol, and stains with borax-carmine. Laid eggs may be killed by Patten's method. After heating, the two lips of the crista of the capsule may be separated with fine

forceps and pieces of the walls torn away, and the eggs pushed out of the compartments formed by their choria and hardened as desired. Good results are also obtained by heating to 80° C. for ten minutes in liquid of Kleinenberg, and preserving in 70 per cent. alcohol. This causes the envelopes to dilate and stand off from the surface of the egg, so that they can easily be dissected away.

HEYMONS (*Zeit. wiss. Zool.*, liii, 1892, p. 434), for young embryos, incises the cocoon at the end by which it adheres in the body of the mother, brings it for two minutes into water heated to 90° C., and opens in Flemming, in which the embryo is dissected out.

MORGAN (*Amer. Natural.*, xxii, 1888, p. 357) puts ova of *Periplaneta* for thirty minutes or an hour into *eau de Javelle* diluted with 4 to 8 volumes of water and slightly warmed, which softens the capsules.

853. Coleoptera. HIRSCHLER (*Zeit. wiss. Zool.*, xcii, 1909, p. 628) fixes ova of *Donacia* (after incising the chorion) for two to three hours in equal parts of sublimate of 6 per cent. and nitric acid of 3 per cent.

GATENBY (*Quart. Journ. Mic. Sci.*, 1917) for Donacia uses Petrunkewitsch or picro-nitric. In the latter case the chorion must be incised.

SALING (*Dissert. Marburg*, 1906, p. 10) fixes ova of *Tenebrio* for about two minutes in a hot mixture of 40 parts of alcohol of 96 per cent., 4 of nitric acid, and 50 of saturated aqueous sublimate; or for three minutes in a hot mixture of 1 part of formol with 3 of water.

KARAWAIEW (*Biol. Centralb.*, xix, 1899, p. 124) kills larvæ of *Anobium* in hot water, freezes them with ether spray, cuts away a lateral strip, lets them thaw, and puts for twenty-four hours into picro-sulphuric acid.

854. Phalangida. The ova of *Phalangium opilio* possess a chorion covered with yellow corpuscles that render them opaque. BALBIANI puts them into water with a few drops of caustic potash, and raises to boiling-point. The ova are then laid on filter-paper, and the chorion removed by rubbing with a camel's hair brush, the vitelline membrane remaining intact, so that the embryo can be studied through it.

HENKING's method (*Zeit. wiss. Zool.*, xlv, 1886, p. 86). Fix with boiling water or Flemming. Preserve the ova in 90 per cent. alcohol. To open the chorion, bring them back into 70 per cent. alcohol, which causes them to swell up so that the chorion can easily be pierced with needles, and the ovum turned out.

855. Araneida. KISHINOUYE (*Journ. Coll. Sci. Imp. Univ. Japan*, iv, 1891, p. 55; *Zeit. wiss. Mik.*, ix, 1892, p. 215) fixes in water warmed to 70° or 80° C., puts into 70 per cent. alcohol,

and after twenty-four hours therein pierces the membranes and passes through stronger alcohol.

See also Locy, *Bull. Mus. Comp. Zool. Harvard*, xii, 3, 1886. Fix by hot water. The liquid of Perényi may also be used; it has the advantage of not making the yolk so granular.

Montgomery (*Journ. Morph.*, xx, 1909, p. 628) fixes ova of *Theridium* for one or two hours in Carnoy and Lebrun's mixture.

Lambert (*ibid.*, p. 420) fixes ova of *Epeira* in picro-sulphuric acid warmed to 70° or 80° C.

Purcell (*Quart. Journ. Micr. Sci.*, liv, 1909, p. 7) fixes ova of *Atta* in boiling saturated sol. of sublimate in alcohol of 70 per cent.

Hamburger (*Zeit. wiss. Zool.*, xcvi, 1910, p. 3) fixes ova of *Argyroneta* in Gilson's mixture.

856. Limulus. Kingsley (*Journ. Morph.*, vii, 1892, p. 38) kills ova by heating in sea-water to 70° or 75° C. and brings into alcohol of 30 to 70 per cent. Similarly Kishinouye, *Journ. Coll. Sci. Japan*, v, 1892, p. 56.

857. Decapoda. Reichenbach (*Abh. Senckenberg Ges. Frankfurt*, xiv, 1886, p. 2) fixes ova of *Astacus* in water gradually warmed to 60° or 70° C. (if the chorion should burst, that is of no consequence), hardens for twenty-four hours in 1 to 2 per cent. bichromate of potash or 0·5 per cent. chromic acid, washes out for the same time in running water, and brings into alcohol. Remove the chorion, and remove the embryo from the yolk with a sharp knife.

Herrick (*Bull. U.S. Fish. Comm.*, xv, 1896, p. 226) kills the ova in hot water, shells and fixes in picro-sulphuric acid.

858. For *Homarus*, see Waite, *Bull. Mus. Comp. Zool.*, xxxv, 1899, p. 155.

859. Amphipoda. Della Valle (*Fauna u. Flora Golf. Neapel*, xx, Monog, 1893, p. 170) puts ova of *Orchestia* by means of a pipette into boiling, cold-saturated sublimate solution, removes them instantly into sea-water, and thence into weak alcohol. If the chorion does not burst of itself it must be pricked with a needle.

860. Cladocera. Haeker (*Zellen. u. Befruchtungslehre*, 1899, p. 60) fixes females of *Sida* with winter eggs in a hot mixture of 100 c.c. alcohol of 70 per cent. with 1 to 2 c.c. saturated sol. of sublimate. See also Samter, *Zeit. wiss. Zool.*, lxviii, 1900, p. 176.

861. Copepoda. Krueger (*Arch. Zellforsch.*, vi, 1911, p. 173) fixes ovaries of Harpactida in Zenker's mixture with 10 per cent. of formol added. No other liquids give good results.

VERMES

862. Rotatoria. Jennings (*Bull. Mus. Harvard Coll.*, xxx, 1896, p. 101) finds the best fixative for pregnant females is the

strong liquid of Flemming, but the ova must then be bleached with chlorate of potash (§ 611).

LENSSEN (*La Cellule*, xiv, 1898, p. 428) fixes ova of *Hydatina* with sublimate for twenty seconds.

863. Turbellaria. GARDINER (*Journ. of Morph.*, xi, 1895, p. 158) finds the best fixative for ova of *Polychoerus* is a mixture of equal parts of absolute alcohol and glacial acetic acid.

BRESSLAU (*Zeit. wiss. Zool.*, lxxvi, 1904, p. 219) fixes Mesostomidæ with summer-eggs in Tellyesniczky's mixture (either cold or warmed to 60° or 70° C.) for ten to twelve hours, and washes out for the same time. He incises winter-ova at one pole, fixes and brings into alcohol of 95 per cent., then makes an incision at the other pole, and imbeds in paraffin through cedar oil. In the paraffin, slices of the shell may be removed with a scalpel, and the ova re-imbedded when sufficiently shelled.

VAN DER STRICHT (*Arch. Biol.*, xv, 1898, p. 370) finds that ova of *Thysanozoon* will only cut well when they have been not more than two minutes in absolute alcohol followed by chloroform and paraffin as used by Carnoy and Lebrun, § 825.

See also, for Polyclads, FRANCOTTE, *Arch. Zool. Expér.*, vi, 1898, p. 196; and, for fresh-water Planaria, IIJIMA, *Zeit. wiss. Zool.*, xl, 1884, p. 359.

864. Cestoda (v. BENEDEN, *Arch. Biol.*, ii, 1881, p. 187). Ova of *Tœnia* in which a chitinous membrane has formed around the embryo are impervious to reagents. They may be put on a slide with a drop of some liquid and covered. Then, by withdrawing the liquid by means of blotting paper, the cover may be made to gradually press on them so as to burst the membranes, and the embryo may then be treated with the usual reagents.

HASWELL (*Quart. Journ. Micr. Sci.*, liv, 1909, p. 417) fixes ova of Temnocephala in " sublimate alcohol," brings them into 90 per cent. alcohol with iodine added, and thence gradually back into water, softens the shells in weak sodium hypochlorite, washes and imbeds.

865. Trematoda. COE (*Zool. Jahrb., Abth. Morph.*, ix, 1896, pp. 563, 566), for the special study of the excretory system of the Miracidia of *Distomum*, kills with osmic acid, rinses with distilled water, and puts for a couple of days into $\frac{1}{4}$ per cent. solution of silver nitrate.

Egg-capsules may be softened with 5 per cent. caustic potash and then burst open (HECKERT, *Bibl. Zool.*, iv, 1889).

Dr. W. REES WRIGHT (*Annal Trop. Med. and Par.*, 1927) uses Bouin and acetic alum carmine or Heidenhain for rediæ and cercariæ.

866. Nematoda. The ova of *Ascaris megalocephala*, a classical object of study, are one of the most impervious things in the animal kingdom. Years ago FOL related to Lee that he had had

ova segmenting right through absolute alcohol into balsam. BATAILLON (*Arch. Entwickelungsmech.*, 1901, p. 149) has had ova showing living embryos after having been for six months in liquid of Flemming, and found them to remain alive for months after drying for twenty-four hours at 35° C., and mounting in balsam, and for weeks in acids or alkalies.

Doubtless the best fixative yet made known for ova furnished with their capsules will be found to be that of CARNOY and LEBRUN, § 91 (*La Cellule*, xiii, 1897, p. 68). After fixation the ova are carefully brought into 80 per cent. alcohol, in which they are preserved. Imbedding should be carefully done as recommended for the ova of Amphibia (§ 825), but they ought not to remain in the pure paraffin for more than a minute to a minute and a half. But these authors prefer the celloidin method. At least six weeks' soaking in the different strengths of celloidin will be necessary to ensure penetration. They stain with iron hæmatoxylin.

ZUR STRASSEN (*Arch. Entwickelungsmech.*, iii, 1896, p. 29) fixes for twenty-four hours in a mixture of 4 parts 96 per cent. alcohol and 1 part acetic acid, brings into pure alcohol, stains with hydrochloric acid carmine, and brings gradually into glycerin.

Similarly ZOJA (*Arch. mik. Anat.*, xlvii, 1896, p. 218) and ERLANGER (*ibid.*, xlix, 1897, p. 309). Zoja stained with Bismarck brown and examined in dilute glycerin; Erlanger made paraffin sections and stained with iron hæmatoxylin.

KOSTANECKI and SIEDLECKI (*ibid.*, xlviii, 1896, p. 184) employed concentrated sublimate solution, or 3 per cent. nitric acid or mixtures of these two, for ovarian ova.

VAN BENEDEN and NEYT (*Bull. Acad. Belg.*, 1887, p. 214) took equal parts of alcohol and acetic acid. BOVERI (*Jena Zeit.*, xxi, 1887, p. 423) fixed in his picro-acetic acid, § 100—a clearly inadequate method. GULICK (*Arch. Zellforsch.*, vi, 1911) has "fixed" ova of *Heterakis* for twenty-two hours in one-third saturated picric acid with 3 per cent. of glacial acetic acid, and had them develop in alcohol of 70 per cent. to stages representing a normal development of several weeks.

BORING (*Arch. Zellforsch.*, iv, 1909, p. 121) spreads ova of *Ascaris* on a layer of Mayer's albumen on a slide, sets the albumen with a drop of formol, fixes with 4 parts of alcohol to 1 of acetic acid, stains in alcoholic hydrochloric acid carmine, and mounts in glycerin.

ARTOM (*Zeit. wiss. Mik.*, xxv, 1908, p. 5) freezes segments of the uteri of *Ascaris* in salt water, and cuts them with the freezing microtome into discs 30 μ thick, and fixes these with divers liquids.

CERFONTAINE (*ibid.*, xxix, 1912, p. 305) brings fixed ova from alcohol into absolute alcohol with 1 per cent. of clove oil, evaporates this down to one-tenth, puts into absolute alcohol with 5 per cent. of clove oil, evaporates again down to one-

tenth, then into the same with 5 per cent. of collodion added, evaporates almost entirely away, and passes through cedar oil into paraffin.

For *Mitochondria* and *Golgi apparatus* it is necessary to treat uteri as does Artom (above explained), and then fix in the proper fluid.

867. ECHINODERMATA, CŒLENTERATA AND PORIFERA

See the chapter on " Zoological Methods."

CHAPTER XXXIV

BLOOD AND GLANDS *

BLOOD

868. Fixing and Preserving Methods. The school of Ehrlich used to fix by *heat*. A film of blood was spread on a cover-glass and allowed to dry in the air, and then fixed by passing the cover a few times, three to ten or twenty, through a flame, or by laying it *face downwards* on a hot plate kept for several minutes or as much as two hours at a temperature at which water not only boils, but assumes the spheroidal state (110° to 150° C.). For details, see GULLAND (*Scottish Med. Journ.*, April, 1899, p. 312 ; RUBINSTEIN, *Zeit. wiss. Mik.*, xiv, 1898, p. 456 ; *Zielina, ibid.*, p. 463). At the present day heat fixation is only used as a preliminary to staining with the Ehrlich-Biondi mixture, § 322.

In wet methods either blood is *mixed at once*, on being drawn, with some fixing and preserving medium, and studied as a fluid mount, or films are prepared and put into a fixing liquid *before they have had time to dry*, or after *drying in the air without heat* for a few seconds (at most ten to thirty).

To *make a film*, place a *very small* drop of blood on the convex side of a perfectly clean slide. Bring down on the slide the edge of another slide (or No. 2 coverslip) held over it at an angle of 45 degrees ; move this along until it touches the edge of the drop and the blood runs along the angle between the two slides. Then move the second slide away from the drop, pressing lightly on the first slide, and the drop will follow it and be drawn out into a film without being crushed. Well-spread films should show the red cells almost touching but with no overlapping. Similarly with two cover-glasses, to make a cover-glass film, which can be floated face down on to fixing or staining liquids in a watch-glass.

Most of the usual fixing agents are applicable to blood. But it is often necessary to employ only such as are favourable to certain stains. Those most recommended in this respect are alcohol, formol, sublimate (should not be too strong), osmic acid in very light fixation, or absolute methyl alcohol, which is an energetic fixative of dried films.

Air-dried films ought to be fixed by prolonged heating or corrosive sublimate before putting into aqueous or glycerin

* By E. S. D.

stains, else they will wash off; but this is not necessary for alcoholic stains.

869. Films of Bone Marrow. A small piece of bone marrow may be teased in serum on a slide. Afterwards this is spread on a slide as in the case of blood films, and stained by one of the Romanowsky stains. MALLORY (*Pathological Technic*, 1924) recommends that the film be fixed for one minute, while still wet, with methyl alcohol or one of the usual fixatives, after which staining is carried out. PRICE-JONES (*Journ. of Path. and Bact.*, 1910, xiv, p. 218) uses the following: Small pieces are transferred to a watch-glass containing a dissociating reagent where a more or less complete emulsion results and cellular elements of the tissue are dissociated. The dissociating solution consists of glycerin diluted with ammonia-free distilled water to form a 10 per cent. neutral solution, titrating against N/10 NaOH and using phenolphthalein as indicator. The initial acid reaction should vary from + 0·1 to + 0·5 Eyre's scale and the reagent has a specific gravity of 1·029 at 15·7° C. A loop-full of this glycerin solution is placed on a coverslip and to this is added a loop-full of the emulsion in the watch-glass and very gently spread over the surface of the slip. The film thus prepared is allowed to dry in the air without heating until a uniform ground-glass appearance is produced. Treat as a blood film with Jenner and after a thorough washing in ammonia-free distilled water and complete drying in air mount in xylol, balsam.

870. Fixing and Preserving in Bulk. Most morphologists are agreed that by far the most *faithful* fixing agent for blood corpuscles is osmic acid. A drop or two of blood (Biondi recommends 2 drops exactly) is mixed with 5 c.c. of osmic acid solution, and allowed to remain in it for from one to twenty-four hours. As a rule, the osmic acid should be strong—1 to 2 per cent. Fixed specimens may be preserved for use in acetate of potash solution (MAX FLESCH, *Zeit. wiss. Mik.*, v, 1888, p. 83).

GRIESBACH also (*ibid.*, 1890, p. 328) combines the osmic acid with certain stains. He mentions methyl green, methyl violet, crystal violet, safranin, eosin, Säurefuchsin, rhodamin and iodine in potassium iodide.

ROSSI (*ibid.*, vi, 1889, p. 475) advises a mixture of equal parts of 1 per cent. osmic acid, water, and strong solution of methyl green, permanent mounts being made by means of glycerin cautiously added.

EWALD (*Zeit. Biol.*, xxxiv, 1897, p. 257) mixes 3 to 4 drops of blood of amphibia or reptiles with 10 c.c. of a solution of 0·5 per cent. osmic acid in 0·5 per cent. salt solution (for mammals 0·6 to 0·7 per cent. salt), siphons off the supernatant liquid after twenty-four hours with his capillary siphon and substitutes water, alum, carmine, etc., and lastly, 50 per cent. alcohol.

WEIDENREICH (*Arch. mik. Anat.*, lxxii, 1908, p. 213) lays a cover with a drop of blood on it on a layer of agar-agar (1 per cent. in salt solution of 0·8 per cent.) and after five minutes runs in osmic acid of 1 per cent., and after five minutes more removes the cover.

Dekhuyzen (*Anat. Anz.*, xix, 1901, p. 536) recommends a mixture of either 3 or 9 volumes of 2 per cent. osmic acid with 1 of 6 per cent. acetic acid, containing ⅛ per cent. of methylen blue, which he calls "Osmacet."

The mercurial liquids of Pasini (§ 453) used to be considered good. Hayem (*Du Sant*, etc., Paris, 1889; see also *Zeit. wiss. Mik.*, vi, 1889, p. 335) has the following formula:—Sublimate 0·5, salt 1, sulphate of soda 5, and water 200. This should be mixed with blood in the proportion of about 1 : 100. Eosin may be added to it. Lowit's formula (*Sitzb. k. Akad. Wiss. Wien.*, xcv, 1887, p. 144) consists of 5 c.c. cold saturated sublimate solution, 5 grm. sulphate of soda, 2 grm. salt, and 300 c.c. water. Mosso finds that both of these are too weak in sublimate.

Duboscq (*Arch. Zool. Exper.*, vi, 1899, p. 481) uses (for blood of Chilopoda) a solution of acetic acid, copper acetate, copper chloride, osmic acid, thionin, 1 grm. each, water 400, which, mixed with the blood, fixes and stains in about two minutes.

Formol has lately been used. Marcano (*Arch. de Med. Exper.*, xi, 1899, p. 434) mixes fresh blood with a mixture of 100 parts of sodium sulphate of sp. gr. 1·020 and 1 of formol; or with water 85 to 100 parts, sodium chloride 1, and formol 1.

Kizer (*Journ. Roy. Mic. Soc.*, 1900, p. 128) simply mixes 1 drop of blood with 3 of 2 per cent. formalin, and allows to stand for an hour.

Schridde (*Hæmat. Techn.*, Jena, 1910, p. 17) lets blood drop into a mixture of 1 part of formol, 9 of liquid of Müller, and 10 of water, fixes therein for two to four hours at 40° C., filters, washes and brings through alcohol and chloroform into paraffin for sectioning.

871. Fixing and Preserving of Films. Muir (*Journ. of Anat. and Phys.*, xxvi, 1892) makes cover-glass films and drops them into saturated sublimate solution, and after half an hour washes, dehydrates, and passes through xylol into balsam.

Gulland (*Brit. Med. Journ.*, March 13th, 1897; *Scottish Med. Journ.*, April, 1899) makes cover-glass films, and after a few seconds drops them face downwards into a solution of:

Absolute alcohol saturated with eosin . .	25 c.c.
Pure ether	25 ,,
Sublimate in absolute alcohol (2 grm. to 10 c.c.)	5 drops.

After three or four minutes they are washed, stained and mounted in balsam.

For Jenner's fixing and staining method, see next section.

Many recent authors fix wet films with *formol.* Benario (*Deut. med. Wochenschr.*, 1895, p. 572) mixes 1 part of 10 per cent. formol with 9 of alcohol (the mixture must be freshly prepared), and plunges films into it for a minute.

Similarly Gulland, with 1 part of formol to 9 of alcohol.

Similarly Wermel (see *Zeit. wiss. Mik.*, xvi, 1899, p. 50), who combines various stains (methylen blue, eosin, gentian, etc.) with the formol.

Edington (*Brit. Med. Journ.*, 1900, p. 19) exposes films for fifteen to thirty minutes to *vapour of formol* under a bell-jar.

SCOTT (*Journ. of Path. and Bact.*, vii, 1900, p. 131) exposes films to the vapour for about five seconds and drops into absolute alcohol, and after fifteen minutes stains and mounts.

A *short* exposure (thirty seconds) to vapour of *osmium* has also been recommended.

SZECSI (*Deutsch. med. Wochschr.*, 1913, p. 1548) has recommended *Lucidol* for blood smears, and smears of fæces containing protozoa and cysts. The formulæ for an acetone and pyridin solution will be found in § **110**, and also of an acetone xylol solution for subsequent washing of the smears.

It is best to keep a sufficient quantity of the fixing solution in staining jars. Make a smear, allow it to dry, and place it in the acetone peroxide of benzol solution for fifteen minutes; transfer to the acetone xylol solution for ten minutes in order to remove the lucidol; wash off in pure methyl alcohol; the slide is now ready for staining. It will be found that most of the current stains used for such smears will act successfully after the lucidol fixation. Pappenheim's panoptic method (§ 879) is recommended.

For smears of fæces a fixation of twenty minutes in pyridin benzol peroxide solution is used; wash as above, in acetone xylol, or pyridin xylol, and then in methyl alcohol.

Possibly the substitution of pure acetone for the methyl alcohol bath might prove advantageous in some ways.

In stains of the Romanowsky group, Jenner, Leishmann and Wright, fixation is accomplished by the methyl alcohol of the undiluted stain. Preliminary fixation is necessary in the case of Giemsa staining. Pure absolute methyl alcohol is to be regarded as one of the most satisfactory fixatives for dry blood films. Two to five minutes is sufficient. Absolute ethyl alcohol may be used but is not so good. The fixation time should then be greatly increased. Thirty minutes or more.

872. Stains of Blood. Fresh unfixed blood may be stained by some dye contained in a diluting fluid, or by a dye deposited on the surface of a slide in which the liquid blood is later placed. The former method is largely used in the enumeration of leucocytes, their nucleus being stained by some dye such as gentian violet in the following commonly used solution given by PRICE-JONES (*Clinical Hæmatology*, 1933), in which the cells are both fixed and stained: 0·8 per cent. sodium chloride, 90 c.c.; 40 per cent. formol, 10 c.c.; gentian violet, a few drops. Supravital staining of neutral red bodies or of mitochondria in leucocytes is carried out by the latter method. SIMPSON (*Journ. Med. Res.*, 1922, xl, p. 77) makes saturated solutions for neutral red and Janus green B in 95 per cent. ethyl alcohol. Slides are prepared by dipping in the mixture, after which they are allowed to dry. A drop of blood is placed on a coverslip, mounted on a prepared slide and ringed with paraffin. The glassware should have an even surface, and

the drop should not be too thick or the dye unevenly spread. Neutral red bodies and mitochondria are stained.

SABIN (*Bull., Johns Hopk. Hosp.*, 1923, xxxiv, p. 277) uses similar saturated solutions, but adds 0·4 c.c. of the neutral red to 10 c.c. of absolute alcohol and 3 drops of Janus green to every 2 c.c. of this solution.

LIGHTWOOD, HAWKSLEY and BAILEY (*Proc. Roy. Soc. Med.*, 1935, xxviii, p. 405) make a 0·25 per cent. solution of Gurr's vital neutral red chloride, and a 0·4 per cent. solution of Gurr's vital Janus green B. Before use 1·75 c.c. of the neutral red and 0·07 c.c. of the Janus green are added to 10 c.c. of absolute alcohol. The slides and coverslips should be perfectly clean.

(See also *Journ. of Exper. Med.*, 1930, lii, p. 279, and CUNNINGHAM and TOMKINS, *Folia. Hæmat.*, 1930, xlii, p. 257.)

Early observers have used this technique for other dye stuffs. LEVADITI (*Journ. Phys. Gen.*, Paris, 1901, p. 245) allows solution of *Brillantkresylblau* in alcohol to dry on a slide, puts a drop of blood on the dried layer, and covers. Similarly CESARIS-DEMEL (*Arch. path. Anat.*, 1909, p. 92), with a mixture of this dye and Sudan III; and NAKANISHI (*Centralb. Bakt.*, 1901, p. 98), with methylen blue BB.

873. Fixed films may be treated with the usual tissue stains, *eosin* being an important one, as it stains rose-red all parts of blood cells that contain hæmaglobin. EHRLICH's acid hæmatoxylin, with 0·5 gr. of eosin dissolved in it, is a good general stain. Or, stain with hæmalum, and then with eosin (0·5 per cent. in alcohol or water).

EHRLICH's triacid (§ 323) gives good general results, and demonstrates neutrophilous granules. His mixture for eosinophilous cells has been given (§ 339).

PAPPENHEIM's panoptic triacid (on sale by Grübler) is Ehrlich's triacid with methylen blue in place of the methyl green.

CHENZINSKI's mixture, which is good, has been given (§ 341). Stain for six to twenty-four hours in a stove. This gives rise to precipitates. To avoid them (WILLEBRAND, *Deutsch. med. Wochenschr.*, 1901, p. 57) you may make a mixture of equal parts of 0·5 per cent. solution of eosin in 70 per cent. alcohol and saturated solution of methylen blue in water, and add acetic acid of 1 per cent. drop by drop till the mixture begins to turn red, and filter before use. Or (MICHAELIS, *ibid.*, 1899, No. 30) make (*a*) a mixture of 20 parts 1 per cent. aqueous methylen blue with 20 of absolute alcohol, and (*b*) a mixture of 12 parts 1 per cent. aqueous eosin with 28 of acetone, and for staining mix equal parts of these and stain for half a minute to ten minutes.

874. JENNER (*Lancet*, 1899, No. 6, p. 370) mixes equal parts of 1·2 to 1·25 per cent. water soluble eosin (Grübler's) and 1 per cent. methylen blue, filters after twenty-four hours, washes the

precipitate on the filter, dries it, and dissolves it in 200 parts of *absolute* methyl alcohol (the solution can be had ready made from Grübler or Hollborn). (Or, simply mix 125 c.c. of 0·5 per cent. solution of the eosin in methyl alcohol with 100 c.c. of 0·5 per cent. solution of methylen blue). Cover-glass films are floated on to this or smears are immersed and stained for four minutes. Another method is to fix the film in the undiluted Jenner stain for one minute, after which it is stained for three minutes in stain to which two volumes of distilled water have been added. Wash off the stain with distilled water until a faint pink colour appears; dry and examine. Erythrocytes red, all nuclei blue, parasites blue, but with unstained nuclei. The May-Giemsa stain is prepared in an almost similar manner, and is used in the same way.

Jenner's Stain for Sections. The following method for sections, proposed by TURNBULL (*Journ. of Path.*, 1931, p. 34), gives good results in formol fixed material, and is widely used. Pieces are fixed for as short a time as is compatible with complete fixation in 4 per cent. neutral saline formaldehyde, or in the same buffered to *p*H7 or to *p*H5. Paraffin sections are rinsed in distilled water and stained with one part of a stock solution of Jenner to one part of distilled water for about forty-five minutes. They are then differentiated with absolute alcohol, and mounted in Gurr's neutral mounting medium. During differentiation they may be cleared from time to time in xylol for examination. Stock solution is made as follows: Pour 100 c.c. of analytical methyl alcohol upon 0·3 grm. of Gurr's Jenner crystals, leave without shaking, and decant after three hours. The stain should be stored in a glass-stoppered bottle. Distilled water should be boiled and cooled before use.

ASSMANN (*Munch. med. Wochenschr.*, 1906, No. 28; *Das eosin-saure Methylenblau*, Leipzig, 1908, p. 35) treats fresh films for half a minute to three minutes in a Petrie dish with a few drops of Jenner's solution (from Grübler or Hollborn), then pours on 20 c.c. of distilled water with 5 drops of 1/10 per cent. solution of lithium carbonate, leaves for five minutes, rinses in distilled water, dries with blotting paper, and mounts in neutral balsam.

The foregoing mixtures give a stain—seemingly due to the formation of an eosinate of methylen *blue*—in which the nuclei of blood cells are blue and their plasma red to violet. It was made out by ROMANOWSKY (*St. Petersburger med. Wochenschr.*, 1891) that *under certain conditions* mixtures of these two dyes give a stain which is in some respects the inverse of this, blood cells being stained in divers hues, according to their kinds, and any *protozoon parasites* that may be present showing *red* nuclei and *blue* plasma, which greatly facilitates their detection and diagnosis. This reaction appears to be due to the formation of

an eosinate—*not* of methylen blue, but—of *Methylenazur*. The method, only vaguely indicated by Romanowsky, has undergone, at the hands of Ziemann, Zettnow, Nocht, Reuter, Michaelis, Ruge, Maurer, Leishman, Giemsa and others, numerous modifications which have culminated in the establishment of a process worked out by Giemsa as perhaps the most trustworthy and efficient of "Romanowsky" stains. This is as follows:

875. Giemsa's **Azur-eosin Process.** You start with a mixture of eosin with *methylenazur* (instead of methylen blue). This mixture is very troublesome to prepare, and is best obtained ready from Grübler and Hollborn (their "Giemsa'sche Lœsung für Romanowskyfærbung." * *Air-dried films* (*Deutsch. med. Wochenschr.*, 1907, No. 17) are fixed in alcohol or in methyl alcohol (two to three minutes), and dried with blotting paper. They are treated for ten to fifteen minutes with a dilution of 1 drop of the stock mixture to 1 c.c. of water, washed under a tap, dried with blotting paper, and again dried in the air and mounted in balsam, or (preferably) preserved unmounted. All reagents, especially the balsam, must be strictly *free from acid.*

876. Wet films (*ibid.*, 1909, p. 1751) are treated as follows: Fix them for twelve to twenty-four hours in a mixture of 2 parts saturated aqueous solution of sublimate with 1 of absolute alcohol. Wash and treat for five to ten minutes with a mixture of 2 parts of iodide of potassium, 100 of water and 3 of Lugol's solution. Wash and treat for ten minutes with 0·5 per cent. solution of sodium thiosulphate. Wash, and stain as above (changing the stain for fresh after half an hour) for one to twelve hours. Then pass through mixtures of acetone with first 5, then 30, then 50 parts per cent. of xylol into pure xylol, and mount in cedar oil. This process is applicable to *sections.*

Or (*ibid.*, 1910, p. 2476) a slide is placed in a Petri dish and covered with a mixture of *equal parts of methyl alcohol and stock mixture.* After half a minute this is poured off and enough distilled water poured in to cover the slide, and the whole is rocked to mix the two. After three to five minutes, wash in running water, dry, and mount in cedar oil.

By any of these processes nuclei (red) are demonstrated not only in hæmatozoa, but in many bacteria, spirochætæ, coccidia, sarcosporidia, etc.

877. Giemsa's **Method for Sections. Paraffin Sections.** Next to hæmatoxylin and eosin, Giemsa's stain is perhaps the most useful for biologists and pathologists alike. The former, however, are slow to appreciate its value. Not only is it helpful for blood

* To make this up from Grübler's powders, dissolve 3 grm. of Azur II-eosin and 8 decigrammes of Azur II. in 125 c.c. of glycerin and 375 c.c. of methyl-alcohol.

and blood-forming organs, but also for the nervous system, in which it gives the best and most constant coloration of the Nissl bodies. It colours also Rickettsia bodies, bacteria, and various pathological inclusions, such as vaccine bodies, Negri bodies, etc. WOLBACH's (*Journ. Med. Res.*, xli, 1919, p. 76) method, slightly modified, is recommended.

Tissues are fixed in any of the usual mercuric chloride fixatives. Very good results are obtained using Maximow's Zenker formol (§ 78), six hours' fixation being ample. Sections 5 μ in thickness, or less, are passed down to water and are treated with Lugol's solution to remove the sublimate, followed by 0·5 per cent. sodium hyposulphite to remove, in turn, the iodine. They are then washed in distilled water. Stain twelve to twenty-four hours in :

Distilled water	50 c.c.
0·5 per cent. sodium bicarbonate . .	2 drops.
Methyl alcohol, pure	1·5 c.c.
Giemsa's liquid stain (Grübler) . .	1·25 ,,

The addition of methyl alcohol retards or prevents precipitation of the dye. Differentiate in 95 per cent. alcohol, dehydrate, clear and mount in cedar oil or any neutral mounting medium.

If the cytoplasmic structures under investigation are too blue (as may be the case if the material has been preserved in formalin instead of Zenker's fluid) omit the sodium bicarbonate and add a little colophonium to the alcohol used in differentiation, or mordant before staining 2·5 per cent. potassium bichromate, or employ a special stain with more of the Azur II. eosin and less of the Azur II. component. If, on the other hand, they are too red, either treat the tissues before staining with 1 per cent. potassium permanganate, followed by 5 per cent. oxalic acid, or else make up a sample of stain containing a higher concentration of the red constituent (Azur II.). In general the *p*H of both the staining fluid and that used in washing should be 6·8 to 7·1. This may be obtained by using the following buffering solution : KH_2PO_4 1 grm., Na_2 HPO_4 2 grm., distilled water 1 litre. With increased *p*H there is increase in the bluish and decrease in the reddish staining, lower values having the contrary effect.

CANTI (*Journ. of Path. and Bact.*, 1935, xl, p. 233) uses Giemsa's solution diluted in this buffer solution in the proportion of 2 drops to 1 c.c. for the staining of virus bodies in tissue cultures. For further information on the effect of hydrogen ion concentration on staining, the papers of MOMMSEN (*Klin. Woch. Jahrg.*, 1926, v, p. 844) ; LA CORTE (*C. R. Soc. Biol.*, lxxxxviii, 1928, p. 1579) ; FRENCH (*Stain Tech.*, vii, 1932, p. 108) ; OCHS (*Folia Hœmat.*, xxvii, 1928, p. 241) should be consulted.

MCNAMARA (*Journ. Lab. and Clin. Med.*, xviii, 1933, p. 752) gives a rapid Giemsa stain for sections.

878. Leishman's **Romanowsky Stain** (*Brit. Med. Journ.*, March 16th and September 21st, 1901) is as follows: To a 1 per cent. solution of Grübler's medicinal methylen blue in water add 0·5 per cent. of sodium carbonate, heat to 65° C. for twelve hours, and let stand for ten days. Then add an equal volume of 0·1 per cent. solution of Grübler's eosin extra B, let stand for six to twelve hours, collect the resulting precipitate on a filter, wash it until the wash comes off colourless, dry and powder. The stain is now prepared by the usual makers. For staining, dissolve 0·15 grm. in 100 c.c. of pure methyl alcohol. Stain films (air-dried) for one to two minutes; flood the film with water for eight minutes, and examine or dry (without heat) and mount in xylol balsam. Nuclei in shades of bluish-red, cytoplasm bluish, parasites blue with ruby red chromatin.

Raadt (*Munch. med. Wochenschr.*, No. 27, 1911; *Zeit. wiss. Mik.*, 1912, p. 236) obtains a Romanowsky stain of blood and parasites with Jenner's solution. Films fixed with alcohol and ether are first stained for five to ten minutes in solution of 1 part methylenblau med. puriss. (Hœchst), 0·5 part of lithium carbonate and 100 of water, kept for at least three weeks and diluted with 10 volumes of water. Rinse with water, dry with blotting paper, flood with Jenner's solution diluted with 2 or 3 volumes of water, and stain for five to ten minutes. Wash, dry with blotting paper, and mount. See also Scott (*Folia Hæm.*, xii, 1911).

Wright (*Journ. Med. Res.*, vii, 1902, p. 138) describes an essentially similar stain which is largely employed by American workers. The powder which is obtainable from the usual makers is usually dissolved in the proportion of 0·15 grm. to 100 c.c. of pure methyl alcohol. Haden (*Journ. Lab. and Clin. Med.*, lx, 1923, p. 64) recommends that after fixation for one minute in the undiluted stain the staining be carried out for four minutes after dilution with an equal part of buffer solution *p*H 6·0 to 6·6. He uses Sørensen's phosphate buffer. The slide is then washed in the buffer solution.

879. Panoptic Staining. Pappenheim (*Folia Hæm.*, 1912, xiii, p. 539) stains films with Giemsa after first fixing and staining with Jenner or May-Grünwald. Air-dried films are fixed for three minutes, after which an equal quantity of distilled water is added and the stain left for one minute. This is poured off and the slide is stained in Giemsa's solution 0·3 c.c. in 10 c.c. of aq. dest., and stained ten to fifteen minutes. This is then washed in distilled water, dried and examined.

Balint (*Klin. Wochenschr.*, i, 1926, p. 147) fixes in Jenner for five minutes, adds 2 c.c. of buffer solution, and stains for ten minutes. He then washes and stains in Giemsa's diluted 1 drop to 1 c.c. of buffer solution for twenty-five minutes. He finds

that a buffer solution of 6·6 to 7·0 gives the best results, and uses Sørensen's phosphate buffer diluted. PAPPENHEIM (*Anat. Anz.*, xlii, 1912, p. 325) proceeds as follows in the case of sections. Bring to distilled water and stain for twenty minutes at 35° C. in May-Grünwald or Jenner diluted 1 in 8 of water. The stain is poured off, and the section stained in Giemsa diluted 0·2 c.c. in 15 c.c. of distilled water for forty minutes at 35° C. After a short differentiation of acetic acid, 5 to 6 drops in 100 c.c. aq. dest., it is washed, blotted and dehydrated in equal parts of acetone and absolute alcohol. Then passed into xylol and mounted in cedar wood oil or neutral balsam. Very good results are obtained with blood-forming organs, granules being well shown.

CAMERON (*Journ. of Path. and Bact.*, xxxv, 1932, p. 933) recommends the panoptic method for staining of the granular blood cells of invertebrates. He notes that the staining power of the eosinophil cells is influenced by the *p*H of the fixative. At *p*H 4·3 it is lost, 5·5 difficult to find, at 7·8 they stain distinctly, and from 9 to 10 there is much fusion.

For a modification of this stain, using methyl green orange, see KARDOS (*Folia. Hæmatol.*, xii, 1911, p. 39).

MAXIMOW (*Ztschr. f. wiss. Mik.*, xxvi, 1909, p. 177) uses a hæmatoxylin Azur II. eosin staining for hæmapoietic organs which is widely used. Material is fixed in his Zenker formol fixative for six hours at body temperature. Two stock solutions of 1 in 1000 eosin Grübler and 1 in 1000 Azur II. Grübler are made. For staining, 10 c.c. of the eosin solution are diluted with 100 c.c. of distilled water and 10 c.c. of the Azur II. solution added. This is stirred and the sections placed in it immediately and stained for six to twenty-four hours. Differentiate in 96 per cent. alcohol until no more colour comes out, abs. alc., xylol and neutral balsam. The results are almost identical with those obtained using the Giemsa stain. In certain cases it may be necessary to vary the proportions of eosin to Azur II. The stock solutions are good for several months.

UGRUIMOW (*Ztschr. f. wiss. Mik.*, xlv, 1928, p. 191) uses stock solutions of Azur II. 1 in 1000 Hollborn and eosin B A extra Hœchst in buffer solution of *p*H 6·3 to 6·6. Before use these are added to 10 c.c. of distilled water of the proportion of 1·5 to 1·6 c.c. eosin to 1 c.c. of Azur II. He stains ten to twelve hours, changing every few hours, and differentiates in acetone, mounting in acid balsam. There are numerous modifications.

880. Restaining Faded Romanowsky Blood Films. Faded films may be restained with iron alum hæmatoxylin as follows: Remove coverslip, if used, with warm xylol solution. Place slides in 90 per cent. alcohol overnight. Bring down to water, place in 4 per cent. iron alum for one hour. Wash lightly and transfer to 1 per cent. hæmatoxylin for three hours. Differentiate

in *acid alcohol* (0·5 per cent. HCl), not in iron alum. Wash in tap-water, upgrade, and mount in balsam or euparal (Gatenby).

881. Staining of Eosinophil Granulocytes. In general the granules of these cells are well stained by the dyes of the Romanowsky series already mentioned. Care should be taken that the *p*H of both the staining solution and of the distilled water used is not on the alkaline side.

The indulin-aurantia-eosin mixture of Ehrlich (§ 339) gives good staining of the granules. Staining may be carried out at 37° C. for four to five and a half hours (McClung, *Microscopical Technique*, 1929).

Biggart (*Journ. of Path. and Bact.*, xxxv, 1932, p. 799) stains eosinophil cells in sections with azo-eosin 1 part in 2000. The material is fixed in Bouin or mercuric chloride and sections are first stained with hæmatoxylin. He also uses the same dilution of Biebrich scarlet after formalin fixation.

882. Mast Cells (Basophil Granulocytes). Numerous methods have been devised for staining specifically the granules of these cells, though they stain readily (usually metachromatically) with any basic dyes of the anilin series except pure methyl green. Unna (*Enzyk. mik. Technik.*, ii, 1910, p. 414) recommends that the material be fixed in chemically pure absolute alcohol and sectioned in celloidin. In the same volume (p. 72) he recommends staining three hours to overnight in polychrome methylen blue with a knife-pointful of alum to a watch-glass of stain, rinsing and then alcohol, oil, balsam. Another method consists of staining in polychrome methylen blue for fifteen minutes, rinsing, then ten minutes in glycerin-ether (§ 883), washing thoroughly and then alcohol, oil, balsam.

Ehrlich (*Arch. mik. Anat.*, xii, 1876, p. 263) stains twelve hours in absolute alcohol 50 c.c., water 100 c.c., glacial acetic acid 12·5 c.c., to which dahlia is added almost to saturation. He washes out with alcohol and mounts in resinified turps. These give a specific metachromatic stain on a light ground.

The stain of Dominici (*C. R. Soc. Biol.*, liv, 1902) is widely used for the demonstration of mast cells, 0·3 grm. orange G and 0·25 grm. eosin dissolved in 50 c.c. of distilled water. He stains twenty to thirty minutes, washed in 60 per cent. alcohol and stains in 0·5 per cent. watery toluidin blue or thionin solution. Masson (*Diagnosties de Laboratoire*, 1923) substitutes erythrosin for eosin.

Schäffer (*Centralb. Phys.*, xxi, 1907, p. 258) fixes in 95 per cent. alcohol or 2 parts alcohol to 1 of formol. Stains for half an hour in 0·25 per cent. methylen or toluidin blue or thionin in 70 per cent. alcohol with 1 per cent. hydrochloric acid added.

Levine (*Journ. of Lab. and Clin. Med.*, xiv, 1928, p. 172) fixes in any of the usual fluids and imbeds in paraffin, stains in

1 to 2 per cent. thionin in water for two to three minutes, then dehydrates in absolute alcohol and counterstains in orange G in oil of cloves, xylol, Canada balsam.

BUZARD (*Bull. d'histol. appliq.*, vii, 1930, p. 264) fixes in either alcohol, formol saline, alcohol formol or bichromate formol. The sections are first stained in 1 per cent. aqueous solution of acid fuchsin for thirty seconds, rinsed rapidly in water and treated with a 0·8 per cent. aqueous solution of bromine for four to five minutes in a closed container until violet. Wash in water, differentiate in 95 per cent. alcohol or 1 per cent. hydrochloric acid in alcohol for one to three minutes until light mauve. Clear in xylol and mount in Canada balsam. After differentiation the nuclei may be stained in iron hæmatoxylin before dehydration. The period in absolute alcohol should be as short as possible. Granules are carmine red.

BOLTON (*Journ. of Morph. and Physiol.*, liv, 1933, p. 549) uses the ethyl violet Biebrich scarlet stain of Bowie (§ 336) for staining mast cells in fishes after Zenker fixation. BATES (*Anat. Rec.*, lxi, 1934, p. 231) finds that in mammals watery fixatives are useless and fixes in methyl alcohol followed by Jenner or cresyl violet.

883. Plasma Cells, Unna's Later Methods. (UNNA, in *Enzyk. mik. Technik.*, ii, 1910, p. 411.)

A. For Large Plasma Cells

1. Ten minutes in Grübler's polychrome methylen blue solution, wash and drain. Fifteen minutes in 1 per cent. orcein solution (Grübler), *without acid*; absolute alcohol, so long as methylen comes away abundantly; bergamot oil, balsam.

2. Methylen blue as above, two minutes. Wash well. Then two minutes in glycerin-ether mixture * (Grübler) diluted with 4 volumes of water. Wash thoroughly (two to five minutes); absolute alcohol, bergamot oil, balsam.

3. Modification of a method of PAPPENHEIM (*Virchow's Arch.*, clxiv, 1901, p. 111). Ten minutes in the warm, 20° to 40° C. in Grübler's carbol-pyronin-methyl-green mixture. Cool rapidly, by plunging the recipient containing the tissues into cold water. Remove the tissues with a platinum wire and rinse. Clear and mount in any neutral mounting medium.

B. For Small Plasma Cells

4. As No. 2, *supra*, but only half a minute in the glycerin-ether.

5. After removal of the celloidin from the section with alcohol and ether, five minutes in polychrome methylen blue, wash,

* Glycerin-ether $C_6H_{10}\,O_3$ is a glycerin anhydride. It is a differentiating agent for basic dyes. The glycerin-ether mixture in question contains alcohol and glycerin, and can be obtained from Grübler.

dry with blotting paper, dehydrate (about a minute) in a mixture of 2 parts alcohol to 3 of xylol, then one minute in xylol; then five to ten minutes in alum-anilin (prepared by allowing anilin to stand over a layer of powdered alum a couple of fingers deep); xylol, balsam.

6. As No. 3, *supra*, after a foregoing stain of two minutes in polychrome methylen blue.

See also Ehrlich in *Virchow's Arch.*, clxv, 1904, p. 198.

884. Blood Platelets of Bizzozero. The enumeration of these bodies is of importance to the clinical hæmatologist. They may be stained in films by any of the Romanowsky stains. According to Pappenheim (*Farbchemie*, p. 107), wasserblau is almost a specific for them.

Wright (*Journ. Morph.*, xxi, 1910, p. 274) studied them in tissues, after fixation with formol or sublimate (not Zenker), by staining with a modified Giemsa stain and bringing through acetone and oil of turpentine into turpentine colophonium.* For their enumeration the blood is diluted with some diluting fluid of which there are a great many, the fluid acting as a fixative, anti-coagulant, and usually as a staining solution. A very good fluid is that given by Nicholson (*Laboratory Medicine*, 1934), consisting of brilliant cresyl blue 0·1 grm. sodium citrate, 0·55 grm. sodium chloride, 0·28 grm. formol, 0·1 c.c., and distilled water 50 c.c. A drop of this is placed on the skin and the needle prick made through the drop. A mixture of blood and the staining solution is collected in a paraffin-lined capsule in the proportion of 1 to 5. Staining occurs almost immediately, and a well mixed drop is placed on a ringed slide, covered with a coverslip, and examined by the oil immersion lens. Platelets may be enumerated by counting the number found in proportion to 1000 red cells. An alternative method is to make a film of the mixture in the ordinary way, stain with any Romanowsky stain and count as before. It is important that the blood be mixed quickly with the diluent so as to prevent the platelets settling. All receptacles and pipettes should be paraffin-lined, since the platelets dissolve on glass surfaces.†

885. Reticulated Cells or Reticulocytes. In these red blood cells a reticulum or skein-like structure is brought into evidence by a supravital staining with various anilin dyes. Good results are obtained with whole blood as given in the method in § 870. A film is made on a slide with an alcohol solution of cresyl blue (0·3 per cent. is sufficient) and allowed to dry. A drop of fresh

* Details *loc. cit.* or *Journ. Roy. Mic. Soc.*, 1910, p. 783. This method demonstrates the megacaryocytes. See also Downey, *Folia Hœmatol.*, xv, 1913, p. 25, for modifications.

† See also Deghwitz, *Folia Hœmatol.*, xxv, 1920, p. 153; Rees and Ecker, *J. Am. Med. Assoc.*, lxxx, 1923, p. 621; and Gradwohl, *ibid.*, cv, 1935, p. 1030.

blood is placed on the slide and covered with a cover-glass when the reticulum in the reticulocytes becomes coloured intensely. (See also CUNNINGHAM, *Arch. Int. Med.*, xxvi, 1920, p. 405.) Good staining is obtained by using the platelet diluting fluid of Nicholson (§ 884). Their enumeration is carried out in exactly the same manner, counting about 200 cells. Films may be made as before and stained by a Romanowsky stain. Reticulocytes are now known to be young corpuscles which have recently lost their nucleus and as such are evidence of blood regeneration. The reticulum is not found in dry or fixed blood preparations and is probably a condensation brought about by the dye of some substance present in the protoplasm.*

Various intracellular bodies have been described in the case of red blood cells. GOLGI (*Boll. Soc. Med. Chir. Pavia*, xxi, 1919) describes a body of uncertain significance which is of the nature of a reticular apparatus. CABOT (*Journ. Med. Res.*, ix, 1903, p. 15) describes filaments which are stained reddish by Romanowsky stains. ISAACS (*Anat. Rec.*, xxix, 1925, p. 299) describes a refractile granule in about 1 per cent. of cells, which he regards as evidence of youth. It is easily seen in fresh blood, fresh blood with a supravital stain, unstained dry films, or films stained with any of the usual stains. Stippling in red blood cells is best shown by methylen blue staining. AUB, FAIRHALL, MINOT, REZNIHOFF (*Medicine*, iv, 1925, p. 1) stain in the following mixture: methylen blue, 1 grm.; potassium carbonate, 1 grm.; distilled water, 100 c.c., diluted 1 in 15 parts before use. Staining is carried out for fifteen minutes, after which the film is washed until bluish-green.

886. WEIGERT'S **Fibrin Stain** (*Fortschr. d. Med.*, v, 1887, No. 8, p. 228). Sections (alcohol material) are stained in a saturated solution of gentian or methyl violet in anilin water (§ 357). They are brought on to a slide and mopped up with blotting paper, and a little Lugol's solution is poured on to them. After this has been allowed to act for a sufficient time they are mopped up with blotting paper, and a drop of anilin is poured on to them. The anilin soon becomes dark and is then changed for fresh once or twice. The anilin is *thoroughly* removed by means of xylol, and a drop of balsam and a cover are added. This stain may be applied to celloidin sections without previous removal of the celloidin.†

* For other solutions see COOK, MEYER, TUREEN, *Journ. Lab. Clin. Med.*, xvi, 1931, p. 1224.

† See also the modifications of this method by KROMAYRE (§ 930); BENECKE (§ 965); UNNA (*Monatsch. prakt. Dermat.*, xx, 1895, p. 140); WOLFF (*Zeit. wiss. Mik.*, xv, 1899, p. 310); and one of another sort by KOCKEL (*Centralb. allg. Path.*, x, 1899); SHUENINOFF (*Zlb. Path.*, xix, 1908, p. 6). Fibrin is also well stained by Mallory's triple stain (§ 961), or Heidenhain's azan stain (§ 811).

887. Elective Staining of Erythrocytes (K. OKAJIMA, *Anat. Rec.*, xi, 1917). This stain is based on the fact that the phosphomolybdic acid lake of the alizarin stains, shows a special affinity for hæmoglobin. Fix material in formol, sublimate, chrome, etc. Transfer sections on slide to aq. dest.; mordant in 10 per cent. phosphomolybdic acid solution for thirty seconds to two minutes; wash in water; stain in this mixture for twenty minutes to twenty hours: sodium sulfalizarinate saturated aqueous solution, 100 c.c.; and 10 per cent. phosphomolybdic acid aqueous solution, 30 c.c. (10 to 50 c.c.); wash in water; alcohols, xylol, balsam. Erythrocytes go bright yellow-orange. Counter-staining may be done in Ehrlich's hæmatoxylin.

The "specificity" of this method is open to doubt, but it gives interesting results. See also LEPEHNE (*Zeigl. Beitr.*, lxv, 1919, p. 183).

888. Microchemical Tests for Oxidation Centres in the Cell. Recently certain workers have claimed to be able to locate centres or regions of oxidation in the cell by means of some substance sensitive to free oxygen. Unna's method is to use a solution of rongalit white, which is a solution of the leucobase of methylen blue kept in a state of reduction by excess of rongalit, an absorption product of formaldehyde with sodium sulphite. See UNNA (*Arch. f. mikr. Anat.*, lxxviii, 1911; *ibid.*, lxxxvii, 1915, p. 96); also HIRSCH and BUCHMANN (*Z. Zellforsch.*, ii, 1930, p. 255). DRURY (*Proc. Roy. Soc.*, 1914) has shown that Unna's claim is inadmissable and consequently his theory of staining by oxidation and reduction is not proven. GRAHAM (*Journ. Med. Res.*, xxxv, 1916) claims to have demonstrated by means of H_2O_2 and naphthol that the granules of leucocytes and myelocytes contain a peroxidase of the peroxide type. Two techniques have been developed for the demonstration of these enzymes known as the oxidase and peroxidase tests. The former depends on the synthesis of indophenol blue from α-naphthol and dimethyl-p-phenylen-diamin. Schultz in his oxidase reaction proceeds as follows: Blood and marrow smears fixed in formalin vapour are treated firstly in the α-naphthol solution; prepared by melting 1 grm. of naphthol on the surface of 100 c.c. of aq. dest., and adding potassium hydrate till the naphthol dissolves. After a few minutes in this solution (cooled) the smears are transferred to a 1 per cent. solution of the dimethyl—for the same time—when a blue colour is seen to appear where the oxidases lie. Mount in glycerin jelly, but blue colour fades.

GRÄFF (*Zent. allg. Path.*, xxvii, 1916, p. 313) introduces the following modification which gives preparations permanent for several months, and is applicable to frozen sections: 0·5 grm. α-naphthol is dissolved by boiling in 250 to 300 c.c. of distilled water, and is placed in a brown flask. Excess of α-naphthol

falls to the bottom as the solution cools, and the fluid is good for four weeks. 0·5 grm. dimethyl-p-phenylen-diamin is dissolved in 250 c.c. of distilled water by light shaking, and stored in a brown flask. It is ready for use after twelve to twenty-four hours, and is good for two to three weeks. Before use the solutions are filtered, and are mixed in equal parts in a shallow dish. Sections to be stained are immediately added and are left in for ten to fifteen minutes, the solution being constantly stirred. They are then transferred to distilled water for a short time, and are transferred to Lugol's iodine diluted 1 in 2 for two to three minutes. (Grams iodine is also good.) They are then transferred to distilled water to which a few drops of lithium carbonate have been added, and are left ten to twenty-four hours, until a good blue colour appears. Lastly, they are counterstained with alum carmine in hæmatoxylin eosin, washed in water, and mounted in glycerolgelatin. By these methods the granules of the neutrophil, eosinophil, and basophile cells are stained a dark blue. The reaction is given by the myelocytes, by the more mature myeloblasts, while young forms contain no enzyme. Lymphocytes, lymphoblasts and plasma cells show no reaction, though cells of the mononuclear series are as a rule faintly positive. It is unfortunate that the negative staining of early myeloblasts should render the reaction useless as a distinguishing test in many doubtful cases.

SCHULTZE (*Zent. allg. Path.*, xxviii, 1917, p. 8) employs 1 per cent. solutions of the constituents dissolving the α-naphthol by the addition of caustic potash to the boiling solution. Blood films are first fixed in 40 per cent. formol 1, absolute alcohol 10.

SHAW DUNN (*Journ. Path. and Bact.*, xv, 1911, p. 120) uses equal parts of 1 per cent. α-naphthol and dimethyl-p-phenylen-diamin, and mounts in water glass, when the staining is good for many weeks. He finds that various phenols may be substituted for α-naphthol and that paraphenylen-diamin also works, in which case the reaction is slower. Other workers vary the proportions of α-naphthol and dimethyl-p-phenylen-diamin and the staining times with equally good results. See also GRÄFF (*Ziegl. Beitr.*, lxx, 1922, p. 1). The peroxidase test is largely used in the case of blood films for differentiating between cells of the myeloid and lymphoid series since the reagents are more stable and are more readily obtainable.

SATO and SEKIGA TOHOKU (*Journ. Exper. Med.*, vii, 1926, p. 3) have published the following method. A benzidene solution is prepared by rubbing 0·2 grm. of benzidene with a few drops of water in a mortar. Two hundred cubic centimetres of water at room temperature are added and the solution filtered. To the filtrate add 4 drops of 3 per cent. H_2O_2. A fresh, dry blood film is covered with 0·5 per cent. copper sulphate. This is poured off and

the benzidene solution applied for two minutes. It is washed thoroughly in water and counterstained two minutes with safranin, neutral red or carbol fuchsin and mounted in cedar wood oil. Peroxidase granules a deep blue. In the case of rabbit blood (*ibid.*, x, 1928, p. 293) they recommend that the quantity of H_2O_2 be doubled. *Ibid.* (xi, 1928, p. 1) gives a modification which distinguishes between eosinophil and pseudoeosinophil cells.

The following method given in the monthly *Bull. of the Path. and Bact. Lab. Assists. Assocn.*, ii, 1935, p. 48, gives good results. Films are fixed two minutes in freshly prepared formalin 1 part, 95 per cent. alcohol 9 parts. They are washed in tap-water and stained in a freshly prepared benzidene solution made by dissolving a few granules of benzidene in 10 c.c. of 40 per cent. alcohol and adding 1 drop of H_2O_2 (10 volumes). Stain five to ten minutes, wash films in tap-water and counterstain in 1 per cent. methylen blue for a few seconds.

Cytoplasm of lymphocytes and allied cells blue, red cells green, and cells of polymorph series amber.

889. GLANDS. (See Chapter XXX)

Mucin. While many stains are used for the demonstration of this substance, none of them is to be regarded as being specific, and also the secretory contents of many undoubted mucous cells fail to stain by the usual mucous stains and require special techniques.

Hoyer (*Arch. mik. Anat.*, xxxvi, 1890, p. 310) finds that the mucin of mucous cells and goblet cells stains with *basic* tar colours and with alum hæmatoxylin, but not with *acid* tar colours. He obtained his best results by means of thionin, and good ones with toluidin blue, both of these giving a metachromatic stain—tissues blue, mucin reddish—and also with methylen blue (which is particularly useful for its power of bringing out the merest traces of mucin), safranin, etc.

Tissues should be fixed for two to eight hours in 5 per cent. sublimate solution, and paraffin sections stained for five to fifteen minutes in a very dilute aqueous solution of the dye (2 drops of saturated solution to 5 c.c. of water).

Hyaline cartilage, the jelly of Wharton, and the Mastzellen of Ehrlich give the same reactions with basic dyes as mucin does.

See also Sussdorf (*Deutsche Zeit. Thiermed.*, xiv, pp. 345, 349; *Zeit. wiss. Mik.*, vi, 1889, p. 205); Bizzozero (*Atti. R. Accad. di Sci. di Torino*, 1889–92; reports in *Zeit. wiss. Mik.*, vii, 1890, p. 61; and ix, 1892, p. 219); also Unna (*ibid.*, xiii, 1896, p. 42).

The safranin reaction is not obtained with all brands of the dye; that of Bindschedler and Busch, in Bale, gives it, whilst safranin O of Grübler does not. Unna employs chiefly polychrome methylen blue.

As regards the thionin stain, see Hári (*Arch. mik. Anat.*, lviii, 1901, p. 678).

Bruno (*Bull. Soc. Nat. Napoli*, 1905, p. 220) fixes and stains the skin of the frog in a mixture of 100 c.c. of formol of 1·25 per cent. with 8 c.c. of 1 per cent. solution of thionin. Mucous glands red.

Kultschizky (*Arch. mik. Anat.*, xlix, 1897, p. 8) fixes in his mixture (§ 60), and stains sections either in safranin with 2 per cent. acetic acid, or in a similar solution of *neutral red* (two to three days, washing out with alcohol).

Mayer (*Mitt. Zool. Stat. Neapel*, xii, 1896, p. 303, or 2nd edition) gives the following two formulæ for mixtures that stain exclusively mucus.

890. Mayer's **Mucicarmine** (*op. cit.*, last §). One gramme of carmine and 0·5 grm. of aluminium chloride with 2 c.c. of distilled water, heated over a small flame for two minutes, and made up to 100 c.c., with 50 per cent. alcohol. This gives a stock solution which is as a rule to be diluted for use tenfold with distilled or tap-water.

891. Mayer's **Muchæmatein** (*ibid.*). **Hæmatein** 0·2 grm. aluminium chloride 0·1 grm., glycerin 40 c.c., water 60 c.c. An alcoholic solution may be made by dissolving in 100 c.c. of 70 per cent. alcohol, with or without the additional 2 drops of nitric acid.

Southgate (*Journ. Path. and Bact.*, xxx, 1927, p. 729) proposes the following useful modification in the preparation of mucicarmine. Powdered carmine 1 grm., dry powdered aluminium hydroxide 1 grm. are put into a 500 c.c. flask and 100 c.c. of 50 per cent. ethyl alcohol added. Anhydrous aluminium chloride 0·5 grm., just powdered, is added, and with frequent shaking the flask is placed on a boiling water-bath and boiled for two and a half minutes exactly. It is then cooled under the tap and filtered. This forms a stock solution good for three months. To stain, dilute 1 in 10, which keeps for about twenty-four hours. Stain fifteen to twenty minutes. The anhydrous aluminium chloride used may be yellow in appearance.

Sass (*Stain Tech.*, iv, 1929, p. 127) describes a modification in the preparation of Mayer's hæmalum. Dissolve 50 grm. of alum $Al_2(NH_2)_2$, $4SO_4$ in 1 litre of boiling water. Remove from hot plate and add 1 grm. hæmatoxylin. Add 1 grm. sodium iodate ($NaIO_3$), cool and filter. Stain should be filtered whenever a metallic scum is present. The solution is best when fresh and retains its properties for six months. The slide is transferred from water to the stain, then washed in distilled water followed by tap-water or sodium carbonate 1 per cent., and again in distilled water. An aqueous or alcoholic counterstain may be used.

892. Young secretion granules (premucin granules) are well shown after fixation in Champy's or Regaud's fixatives, staining by iron alum hæmatoxylin or by Altmann's acid fuchsin, as in the Bensley method. Counterstaining with methyl green in the latter technique, stains formed mucin greenish-blue, premucin red. DUTHIE (*Proc. Roy. Soc.*, Series B, cxiv, 1933, p. 20).

Goblet cells give all the reactions above described. See PANETH (*Arch. mik. Anat.*, xxxi, 1888, pp. 133 *et seq.* LIST, *ibid.*, xxvii, 1886, p. 481).

Mucous staining in the cells of the surface epithelium of the stomach and of Brünner's glands will be described in the appropriate sections. See also BENSLEY (*Amer. Journ. Anat.*, ii, 1902, p. 104).

893. Salivary Glands. It must be remembered that the nature of the intercellular secretion product in the cells varies greatly from animal to animal, so that it is not possible to make definite rules. Good preservation of granules is usually obtainable with formalin or any of the mercuric chloride fixatives. Chrome-osmic fixatures such as CHAMPY, PODWYSSOZKI (§ 47) or those of METZNER (*Abd. Hand. de Biochem.*, *Arbeits Methoden*, viii, 1915, p. 185) are often successful. Iron hæmatoxylin or the Bensley-Cowdry stain (§ 701) may show the granules extremely well or may fail completely. The latter should not be used after any mercuric chloride fixative. Basic anilin dyes such as toluidin blue, trypan blue, or thionin are sometimes of value.

See also SOLGER (*Unters. 2 Naturlehre d. Menschen*, xv, 5 and 6, pp. 2–15 ; *Festschr. f. Gegenbaur*, ii, 1896, p. 211) ; KRAUSE (*Arch. mik. Anat.*, xlv, 1895, p. 94 ; *ibid.*, xlix, 1897, p. 709) ; MÜLLER (*Zeit. wiss. Zool.*, 1898, p. 640) ; DUTHIE (*Proc. Roy. Soc.*, Series B, cxiv, 1933, p. 20).

894. Gastric Glands. As in the case of the salivary glands considerable confusion arises owing to staining differences between the homologous gland cells in various animals. Such staining methods as have been devised aim at contrasting the parietal cells (Belegzellen) with the serous and mucous chief cells, sometimes they differentiate between the latter two.

KOLSTER (*Zeit. wiss. Mikr.*, xii, 1895, p. 314) stains with hæmatoxylin followed by acid fuchsin, chief cells blue, parietal cells red. Similar results are obtained by counterstaining with eosin or congo red.

HARVEY (*Amer. Journ. Anat.*, vi, 1907, p. 207), working on the dog, uses Bensley's copper chrome hæmatoxylin as follows, for parietal cell granules. Fix two hours in a mixture of equal parts formalin, 3 per cent. $K_2Cr_2O_7$, saturated mercuric chloride solution, and water. Other fixatives, Kopsch, Bensley, Bouin and Zenker, give poor or no fixation of the granules. Sections are placed one minute in a saturated solution of neutral copper acetate, one

minute in 3 per cent. $K_2Cr_2O_7$, and one minute in saturated aqueous solution of hæmatoxylin, washing in tap-water each time. The round is repeated once and the slide is differentiated in Weigert's borax ferricyanide (§ 1056). The parietal cells are black.

Using the tri-colour stain of Bensley (§ 894) he finds that equal parts of a saturated aqueous solution of acid fuchsin and orange G stain the granules of the parietal cells a definite pink in a minute or two, and if followed by a saturated solution of toluidin blue it gives a pretty metachromatic pink in the surface mucus, and in the thick of the cells of the surface and stomach pits. Zymogen and prozymogen are blue.

Kirk (*Amer. Journ. Anat.*, x, 1910, p. 475), working on the pig stomach, fixes in the Bensley mixture of equal parts 3 per cent. $K_2Cr_2O_7$ and saturated solution of $HgCl_2$ in absolute alcohol (§ 108). He fixes in the dark for half to two hours, after which the tissue is brought through 50 per cent. alcohol and so on to paraffin. He finds that the Bensley tri-colour stain is the best all-round stain. In addition he uses Bensley's neutral gentian and copper chrome hæmatoxylin given by Harvey. See also Ferguson (*Amer. Journ. Anat.*, xli, 1928, p. 403).

Florey and Harding (*Journ. Path. and Bact.*, xl, 1935, p. 212) stain mucus in the surface epithelium of the stomach as follows. Nuclei are first stained at 56° C. for ten minutes in acid carmine. The section is then washed and stained in Ehrlich's anilin oil gentian violet for one minute and washed in Lugol's iodine for one minute. Blot. Differentiate in xylol 4 parts and anilin oil 1 part under the microscope. Blot. Wash in xylol. Rinse in absolute alcohol, xylol. Mucin of superficial cells and goblet cells are an intense blue. Brünner's glands less well stained.

895. The secretory canaliculi of the parietal cells may be demonstrated by Golgi's bichromate and silver method, especially with rejuvenated material. (See Sacerdotti.) Tissue is left for five to six days in half saturated sulphate of copper, and then for twenty-four hours in osmic-bichromate mixture. Imbed rapidly in paraffin. See R. and L. Monti (*Rich. Lab. Anat. Roma*, ix, 1902). The indicators neutral red, cyanamin, and naphthol blue give excellent results in the supravital coloration of the contents of the ducts and canaliculi. (Harvey and Bensley, *Biol. Bull.*, xxiii, 1912, p. 239.)

896. Intestine. Florey and Harding (*Journ. Path. and Bact.*, xl, 1935) use the eosin-aurantia-indulin mixture of Ehrlich (§ 339) for staining of Brünner's glands. Goblet cells are unstained, but Brünner's gland cells and pyloric gland cells are blue-green.

897. Paneth Cells. The granules of these cells stain well with eosin, iron hæmatoxylin, or acid fuchsin after most fixations. Klein (*Amer. Journ. Anat.*, v, 1906, p. 323) working on the guinea-pig fixes in equal parts of alcoholic sublimate and Kopsch's

fluid and stains in Bensley's neutral gentian or in iron hæmatoxylin. Better results are obtained with Bensley's tri-colour stain as follows. Sections are stained one minute in equal parts of a saturated aqueous solution of orange G and acid fuchsin, washed in water, and then stained one minute in a saturated aqueous solution of toluidin blue. Wash in water, absolute alcohol, and mount in balsam : chromatin and prozymogen (basal filaments), intense blue, protoplasm a faint bluish, zymogen red. Goblet cells unstained. See KULL (*Arch. mikr. Anat.*, lxxvii, 1911, p. 541).

898. Argentophil Cells. MASSON (*Diagnostics de Laboratoire*, 1923, p. 701) stains the granules of these cells as follows. Frozen sections of formol fixed material are washed two to three hours in water containing 10 drops of NH_3 per litre until all traces of formol or chloride are eliminated. They are placed in stoppered vessels containing Fontana's fluid in the dark for thirty-six to forty hours. Specificity is lost if the time be exceeded. Celloidin or paraffin sections may be treated in the same way, though a longer period in the liquid is usually necessary. The sections are then washed in distilled water, Cajal's toning bath for ten minutes, rinsed in water and washed in hyposulphite solution for one minute. Wash in running water fifteen minutes. In well-stained sections the granules alone appear black. Fontana's liquid is prepared as follows. Ammonia is added drop by drop to a 5 per cent. solution of silver nitrate until the silver oxide formed is just redissolved. More 5 per cent. silver nitrate is now added until there is a permanent opalescence in the liquid which should not smell of ammonia. Cajal's toning bath is as follows. Solution A is 2 per cent. ammonia sulphocyanate in water 3 c.c., $Na_2S_2O_3$ 3 grm., water 100 c.c. Solution B is a 1 per cent. solution of gold chloride. The bath is a mixture of equal parts of the two solutions made immediately before use.

899. Liver. Ordinary histological and cytological fixatives give excellent results.

For lattice fibrils see OPPEL (*Anat. Anz.*, i, 1890, p. 144 ; vi, 1891, p. 168) puts pieces of liver or spleen (alcohol material) for twenty-four hours into a solution of neutral chromate of potash ($\frac{1}{2}$ to 10 per cent.), then for twenty-four hours into a $\frac{3}{4}$ per cent. solution of silver nitrate, washes, dehydrates and cuts without imbedding. The lattice fibres are only stained near the surface, so that tangential sections must be made.

Similarly BERKLEY (*ibid.*, 1893, p. 772) fixing in picric acid, then in an osmium bichromate mixture, and then silvering.

The silver methods of Bielschowsky and Rio Hortega (§ 1022) give good results.

For bile capillaries BRAUS (*Deutsch. Med. Nat. Ges. Jena*, v, 1896, p. 307) uses the rapid method of Golgi hardening in a

mixture of 1 part formol with 3 parts liquid of Müller or $\frac{1}{3}$ per cent. chromic acid; CIECHANOWSKI (*Anat. Anz.*, xxi, 1902, p. 426) uses Weigert's myelin stain (1885 method), EPPINGER (*Ziegl. Beitr.*, xxxi, 1902, p. 230) hardens five to ten days in 10 per cent. formol, and transfers for ten days to Weigert's gliabeize (§ 1083) at room temperature or for five days at 37° C. One may also mix this with the formol in fixation (11 parts beize to 1 part formol). This is preferable in the case of fresh material. Pieces are then washed in water, hardened in alcohol and imbedded in celloidin. Sections are placed in 1 per cent. hæmatoxylin (fresh hæmatoxylin twenty-four hours, old solutions fifteen minutes). He then transfers to a concentrated solution of copper-acetate in water for five minutes and to distilled water for an indefinite period (one to two days if desired). The tissue is then differentiated in Weigert's borax ferricyanide solution (diluted 1 in 5 to 1 in 9). The undiluted solution is used only with over-stained sections. After well washing he transfers to a concentrated solution of lithium carbonate until the brown staining of the celloidin is lost. Sections are then well washed and mounted.

OTAMI (*Proc. New York Path. Soc.*, xxvi, 1926, p. 2) tans paraffin sections of material not fixed in either alcohol or Kaiserling's fluid, for one to two hours in a saturated solution of potassium bichromate at 37° C. Washes five to ten seconds in distilled water and stains five to sixty minutes at 37° C. in Kultschitzky's hæmatoxylin (§ 1059). This is then differentiated in Weigert's borate solution (§ 1056), passed through alcohol into xylol and mounted in balsam. The bile ducts are blue to brown. Results are variable.

McINDOO (*Arch. Path.*, vi, 1928, p. 598) uses the following modification of the Rio Hortega silver carbonate stain (Fontana). Small blocks are fixed at least twenty days in 10 per cent. formaldehyde, and frozen sections cut as thinly as possible. Five or six sections are taken and are heated and cooled for twenty minutes in a silver carbonate pyridin bath until uniformly golden brown. Care must be taken that the heating does not reach the boiling-point by ceasing when steam reaches the surface. Rapidly wash in distilled water and place in 20 per cent. neutral formol, one minute after which fixation is carried out in a 20 per cent. neutral solution of formaldehyde for one minute, followed by fixation in 2 per cent. $Na_2S_2O_3$ for a half to one minute. Wash thoroughly two to three days in tap-water to which a little neutral formaldehyde may be added. Then 95 per cent. alcohol, carbol xylol and balsam.

The silver carbonate pyridin bath is

10 per cent. $AgNO_3$, 30 c.c.
Sat. aqueous, L_1CO_3, 30 c.c.

Wash precipitate several times in doubly distilled water,

decanting and discarding the washings. When the precipitate is thoroughly washed, add 100 c.c. doubly distilled water and dissolve half to three-quarters of it with ammonia, added drop by drop. Filter the supernatant fluid with an opaque bottle and store in dark. This stock $A_g(Co_3)_2$ solution keeps two to four weeks.

The bath is the silver carbonate solution 5 c.c., distilled water 5 c.c., and pyridin 2 to 3 drops. The porcelain dish in which the sections are heated should not be washed after use as the silver coating improves impregnation.

900. Spleen. For hæmatological studies this is best fixed in Helly's or Maximow's fluids for not more than six hours. Thin pieces are preferable and perfusion is advisable.

KULTSCHITZKY (*Arch. mik. Anat.*, xlvi, 1895, p. 675) studies the *musculature* in sections (of material from liquid of Müller) stained for a day or more in a solution of lakmoid in ether and mounted in balsam.

For *elastic fibres* he puts sections for half an hour or a day into a mixture of 800 parts 96 per cent. alcohol, 40 parts 1 per cent. solution of carbonate of potash, 2 parts Magdala red, and 1 part methylen blue.

For the *blood vessels* he puts sections of Müller material for a few minutes into a solution of 1 or 2 parts of Säurerubin in 400 parts of 3 per cent. acetic acid, washes out in 2 per cent. acetic acid, and after-stains in a similar solution of helianthin or wasserblau until the red only remains in the erythrocytes.

Reticular (lattice) fibres are best studied by the various silver techniques and their modifications. FOOT (*Anat. Rec.*, xxxvi, 1927, p. 99) fixes in Zenker directly or after perfusion, or in 10 per cent. neutral formol. He impregnates (*a*) by his modification of the BIELSCHOWSKY technique (*Journ. Lab. and Clin. Med.*, ix, 1924, p. 777), (*b*) by his modification of HORTEGA's silver ammonium carbonate technique (*Arch. Path.*, iv, 1927, p. 36), and (*c*) by his modification of ACHÚCARRO's silver tannate method (*Arch. Path.*, iv, 1927, p. 211). The original papers should be consulted.

Probably the simplest and most successful of the silver techniques for reticular fibres is that recently devised by WILDER (*Amer. Journ. Path.*, xi, 1935, p. 817). Tissues are fixed in 10 per cent. formalin, acetic-Zenker or formol-Zenker. Tissues may be imbedded in paraffin, celloidin or cut as frozen sections. Paraffin sections are brought to water, celloidin sections are stained in dishes before mounting, and frozen sections may be stained in dishes or mounted on slides and attached with thin celloidin. They are then treated as follows :—

Pre-treatment. Place the sections in 0·25 per cent. potassium permanganate or in 10 per cent. phosphomolybdic acid for one

minute. Rinse in distilled water and place in hydrobromic (Merck's concentrated, 34 per cent., 1 part; distilled water, 3 parts) for one minute. Hydrobromic acid may be omitted following the use of phosphomolybdic acid.

Sensitisation. Wash in tap water, then in distilled water and dip in 1 per cent. uranium nitrate (sodium free) for five seconds or less.

Impregnation. Wash in distilled water for ten to twenty seconds and place in silver diamino hydroxid (Foot) for one minute :—

To 5 c.c. of 10·2 per cent. silver nitrate add ammonium hydroxid drop by drop until the precipitate which forms is dissolved. Add 5 c.c. of 3·1 per cent. sodium hydroxid and just dissolve the resulting precipitate with a few drops of ammonium hydroxid. Make the solution up to 50 c.c. with distilled water.

Reduction. Dip quickly in 95 per cent. alcohol and reduce for one minute in the following solution :—

Distilled water, 50 c.c.; 40 per cent. neutral formalin (neutralised with magnesium carbonate), 0·5 c.c.; 1 per cent. uranium nitrate, 1·5 c.c.

Toning. Wash in distilled water and place in 1 : 500 gold chloride (Merck's reagent) one minute. Rinse in distilled water. Place in 5 per cent. sodium thiosulphate (hyposulphite) one to two minutes.

Counterstaining and Mounting. Wash in tap water; counterstain, if desired, with hæmatoxylin and Van Gieson, or hæmatoxylin and eosin; dehydrate in alcohol. Clear in xylol and mount in balsam. The use of ammonia must be avoided in blueing sections after hæmatoxylin as it dissolves the silver.

The use of distilled water and clean glassware for all solutions is essential. All the solutions may be used repeatedly and kept in Coplin jars for several days. The solutions keep without disintegrating in amber glass-stoppered bottles for an indefinite time.

Another variant is that of Gordon and Sweets (*Amer. Journ. Path.*, xii, 1936, p. 545). It is claimed that this method gives impregnation of the finest fibres, while not requiring precise or rapid changes of the sections from one solution to another.

1. Fix in 10 per cent. aqueous formalin or in Bouin's solution. 2. Cut frozen sections or imbed blocks in paraffin or in celloidin. Affix frozen or paraffin sections to slides by Wright's technique or by Mason's gelatin glue method, or ensheath in celloidin by Warthin's molasses-celloidin sheet method. 3. Oxidise for one to five minutes in acidified permanganate solution: 47·5 c.c. of 0·5 per cent. aqueous potassium permanganate plus 2·5 c.c. of 3 per cent. sulphuric acid. 4. Wash in water. 5. Bleach until white in 1 per cent. oxalic acid. 6. Wash in tap water and two

changes of distilled water. 7. Mordant for fifteen to thirty minutes (or longer) in 2·5 per cent. aqueous iron alum. 8. Wash in two or three changes of distilled water. 9. Impregnate for a few seconds in diamino silver hydroxide. To 5 c.c. of 10·2 per cent. aqueous silver nitrate solution add strong ammonium hydroxide solution, drop by drop, until the precipitate is just dissolved. Add 5 c.c. of 3·1 per cent. sodium hydroxide to the ammoniated silver solution, redissolve the resultant precipitate with a drop or two of strong ammonium solution and dilute to 50 c.c. with distilled water. 10. Wash briefly in distilled water. 11. Reduce in 10 per cent. aqueous formalin. 12. Wash in water. (If the sections are over-impregnated repeat the process from step 7.) 13. Tone in 0·2 per cent. yellow gold chloride one to three minutes. 14. Wash in tap water. 15. Fix in 5 per cent. sodium thiosulphate five minutes. 16. Wash well in tap water. 17. Dehydrate in 80 per cent. and in 95 per cent. alcohol. 18a. For sections affixed by Wright's method. Complete dehydration in absolute alcohol and dissolve celloidin in equal parts of absolute alcohol and ether. 18b. For celloidin or celloidin sheet sections. Complete dehydration in carbol-xylol (xylol 2 parts, phenol 1 part). 19. Clear in xylol. 20. Mount in balsam.

901. Lymphatic Glands. The methods applicable to this spleen are in use with these glands. For reticular fibres especially, see Roessle and Yoshida (*Beitr. path. Anat.*, xlv, 1909, p. 110, or *Zeit. wiss. Mik.*, xxvi, 1909, p. 295). Sections stained with hæmatoxylin and eosin, or Weigert's iron hæmatoxylin, or Bielschowsky's neurofibril stain as applied by Maresch (*loc. cit.*), § 967. The sections should not remain for more than fifteen to thirty minutes in the oxide bath.

902. Kidney. Sauer (*Arch. mik. Anat.*, xlvi, 1895, p. 110) finds that for the renal epithelium the best fixative is Carnoy's acetic alcohol with chloroform (three to five hours, washing out with absolute alcohol). A mixture of 9 parts alcohol with 1 of nitric acid is also good, as is the liquid of Perényi. He stains with iron hæmatoxylin, and after-stains in a very weak solution of Säurerubin in 90 per cent. alcohol, which stains the ciliary plateau. He macerates with iodised serum or one-third alcohol, staining afterwards with dahlia.

McGregor (*Amer. Journ. Anat.*, v, 1929, p. 545), in his study of the glomerulus finds three stains to be the most useful: (*a*) Heidenhain azan carmine, (*b*) Ohmori's reinblau-picric acid, (*c*) Lee-Brown's modification of Mallory's anilin blue. The first of these is a slightly modified form of that given in § 811. The reinblau-picric acid stain of Ohmori (*Virch. Arch.*, ccxxxiv, 1921, p. 53) is as follows: Nuclei are stained for at least thirty minutes in acid fuchsin or lithium carmine, and then for one minute in reinblau-picric acid. This is prepared by adding a concentrated

watery solution of reinblau to a concentrated watery solution of picric acid until a dark green colour appears. Sections are differentiated in absolute alcohol, xylol, balsam.

Mallory's anilin blue (modified by LEE-BROWN, *Journ. Urol.*, xxi, 1929, p. 259) is as follows: Acid fuchsin 1 per cent. for thirty seconds. Distilled water one to two minutes. Mallory's stain:

Anilin blue	0·5 grm.	one to five minutes.
Orange G	2·0 „	
Phosphomolybdic acid . . .	2·0 „	
Distilled water	100 c.c.	

Distilled water two to five minutes. Phosphomolybdic acid 1 per cent. for thirty seconds. Distilled water one to two minutes. Dehydrate, clear mount.

ARNOLD (*Anat. Anz.*, xxi, 1902, p. 417) employs *intra-vitam* staining methods for the study of the granules of the epithelial cells. Sections of fresh kidney are cut with a Valentin's knife, and brought into a very dilute solution of neutral red, or methylen blue, in which the granules stain in a few minutes or hours. Or saturated solutions of the dyes, or of indigo carmine, may be injected subcutaneously during life, at intervals of fifteen to twenty minutes, and after two to five injections the organ may be excised and sections made and examined (see §§ 739 and 775).

For demonstration of the membrana propria, see FRISCH (*Anat. Anz.*, xlviii, 1915, p. 284).

For the micro-chemical demonstration of urea, uric acid, and sodium urate, see SCHULTZ (*Virch. Arch.*, cclxxx. 1913, p. 519).

903. Pancreas. Zymogen granules stain well with eosin, acid fuchsin, or iron hæmatoxylin after the usual fixatives. Good cytological results are obtained by the methods given in § 684. For the Golgi apparatus, both Da Fano's formalin silver nitrate and the Nassanow modification of the KOLATCHEW technique (*Arch. fur. mikr. Anat.*, xcvii, 1923, p. 136; ciii, 1924, p. 437) are in use. The pancreas stains well supravitally or intravitally with neutral red or Janus green. With the former dye the prozymogen granules are stained and later the large Krinom bodies of CHLOPIN (*Arch. fur exp. Zellf.*, iv, 1927, p. 462) are formed. HIRSCH (*Zeit. Zellforsch*, xiv, 1932, p. 517; xv, p. 37). DUTHIE (*Proc. Roy. Soc.*, Series B, cxiv, 1933, p. 20).

904. Methods for Islet Tissue. BENSLEY (*Amer. Journ. Anat.*, xii, 1911). Animal killed by bleeding; a cannula introduced into aorta and a solution of neutral red in isotonic salt solution containing 1 in 15,000 neutral red, is injected. Immediately after the pancreas has assumed a faint rosy tint a part of the organ is removed—the islets of Langerhans stain intense yellow-red, and rest faint rosy-pink. In a short time after mounting the islets remain the only stained elements, owing to bleaching in the acini. Method applicable to the counting of the islets of Langerhans.

Janus Green Method. See § 761. Islets deep blue on a red background.

Pyronin Method for Ducts. Inject a 1 in 1000 solution of pyronin, as above, for neutral red method. The ducts stain intensely red. Double stains may be made by injecting mixed Janus green and pyronin (BENSLEY, *op. cit.*).

Methylen blue, 1 in 10,000, may also be used for this purpose. After injection fix in 5 per cent. ammonium molybdate, for which see also Chapter XVIII.

GRAND-MOURSEL and TRIBONDEAU (*C. R. Soc. Biol.*, liii, 1901, p. 187) recommend for *pancreas* Nicolle's "thionine phéniquée," which stains the insulæ of Langerhans hardly at all, the rest strongly.

LANE'S **Methods for Demonstration of A Cells of the Islets of Langerhans.** 1. Fix tissue for from two to four hours in equal parts of saturated alcoholic solution of mercuric chloride and $2\frac{1}{2}$ per cent. potassium bichromate. Wash in 50 per cent. alcohol, then upgrade and imbed; 3μ sections are stained in neutral gentian, obtained by precipitation of equivalent solutions of gentian violet (crystal violet) and orange G. If the correct quantity of the latter is added to the former, a practically complete precipitation is obtained. The precipitate is soluble in alcohol or acetone. For staining add the stock alcohol solution to 20 per cent. alcohol until a solution having the colour of good hæmalum is obtained. Allow to stand for twenty-four hours. Stain for twenty-four hours, blot, dehydrate in acetone, toluol, differentiate in absolute alcohol 1 part, oil of cloves 3 parts, wash in toluol, and mount in balsam.

2. Fix in 70 per cent. alcohol, then stain in neutral gentian as above.

LANE'S **Methods for Demonstration of B Cells of Islets of Langerhans.** Fix for four to twenty-four hours in :—

$K_2Cr_2O_7$	2·5 grm.
$HgCl_2$	5·0 ,,
Aq. dest.	100·0 c.c.

Dehydrate, clear, imbed, and section; stain in neutral gentian as above.

Formalin Bichromate Method for Fixation. This gives a very regular and reliable fixation, and is suitable where one is carrying out observations which necessitate a successful routine method. BENSLEY (*op. cit*) uses 10 c.c. of neutral formalin to 90 c.c. of Zenker's fluid without acetic acid, for twenty-four hours. Stain in neutral gentian, acid fuchsin and toluidin blue, iron hæmatoxylin or Mallory (§ 961).

HOMANS (*Journ. Med. Research*, xxx, 1914), used Bensley's modified Altmann fixative (OsO_4 of 4 per cent., 2 c.c.; potassium bichromate of 2·5 per cent., 8 c.c.; glacial acetic acid, 1 drop),

Lane's methods (*vide supra*), and ordinary hæmatoxylin and eosin.

Very good results are obtained after this fixative, using the Cowdry-Bensley acid fuchsin methyl green stain. Bensley's 1911 article should be consulted for details of other staining methods.

Bowie (*Anat. Rec.*, xxix, 1925, p. 57) fixes in $HgCl_2$ 5 grm., $K_2Cr_2O_7$ $2\frac{1}{2}$ grm. Glacial acetic acid 2 c.c. and distilled water 100 c.c., made freshly before use. He then stains in a modification of Bensley's neutral gentian in which basic ethyl violet is substituted for gentian violet and biebrich scarlet for orange G, made as follows: Watery solutions of biebrich scarlet (Amer. Aniline Products), and basic ethyl violet (Nat. Aniline Co.) are filtered and mixed in the proportion of one to two. The precipitate is washed in distilled water, dried in warm air, weighed, and a stock solution made in absolute alcohol. Stain for twenty-four hours in a solution of 1 mgm. of the dye in 100 c.c. of 20 per cent. alcohol. Rinse in acetone and differentiate in Bensley's mixture of absolute alcohol 1, oil of cloves 3, controlling under microscope. Staining may be prolonged without harm—α cells are blue, β cells purple, γ cells bright red—different results are obtained if acetic acid be omitted from the fixative.

Bloom (*Anat. Rec.*, xxxix, 1931, p. 363) fixes in Maximow's Zenker fluid and stains in Mallory's azan stain (§ 961), granules of A cells light red, B cells grey orange and D cells blue.

See also Babkin, Rubaschkin and Ssawitsch (*Arch. f. Mikr. Anat.*, Bd. 74; Helly, *ibid.*, Bd. 67); Lane (*Amer. Journ. Anat.*, vii, 1907); Saguchi (*ibid.*, vols. 26 and 28); Clara (*Z. f. mikr. Anat. Forsch*, i, 1924, p. 4); Kolossow (*ibid.*, xxvii, 1927, p. 43).

905. Thyroid. Bensley (*Amer. Journ. Anat.*, 1916, xxix) uses brazilin and water blue. Fix gland in Zenkerformol. Section in paraffin and fix sections to slide with water alone or very little albumen; pass through toluol, absolute alcohol, water, iodise, and place in this brazilin solution for several hours :—

Phosphotungstic acid	1·0 grm.
Distilled water	100·0 c.c.
Brazilin	0·05 grm.

The brazilin is first dissolved in a small quantity of distilled water by the aid of heat and added to the phosphotungstic acid solution. This solution goes bad after three days. After staining in the brazilin, wash in water, and place for five minutes in this mixture :—

Phosphomolybdic acid	1·0 grm.
Wasserblau	0·2 ,,
Water	100·0 c.c.

Wash rapidly in water, dehydrate in absolute alcohol, clear in

toluol, and mount in balsam. Cytoplasm stains blue to lilac, nuclear chromatin deep red, contents of thyroid vacuoles sky blue, and colloid droplets of Hürthle deep blue to deep red.

Williamson and Pearse (*Journ. of Anat.*, lvii, 1923, p. 193) fix and mordant in a special solution made as follows: Potassium bichromate 40 grm., chromium fluoride 40 grm., are dissolved by boiling for half an hour in 2000 c.c. of distilled water. The solution is cooled, filtered, and 100 grm. of mercuric chloride is dissolved by boiling in the filtrate. Pieces less than 3 mm. in thickness are fixed, hardened and mordanted in twelve hours and after washing in water are imbedded in paraffin, celloidin, or gum. The fluid may also be used as a simple mordant after formol, alcohol, or osmic fixatives, but pieces must be first well washed, acetic, formic and osmic acids being inimical to the mordanting. Sections are brought to alcohol and I_2 is used to remove crystals as well as for some action which it exercises. The sections are then transferred to 95 per cent. alcohol for half an hour, and are then brought to water where they are treated with $\frac{1}{4}$ per cent. $KMnO_4$ for ten minutes, followed by 5 per cent. oxalic acid until just decolourised, and are washed in running water for ten minutes. They are then stained in Mallory's phosphotungstic hæmatoxylin (§ 312) for fifteen to twenty-four hours and brought to mounting after washing.

Haver (*Journ. Path. and Bact.*, xxx, 1927, p. 621) fixes in 7 per cent. formol or in a mixture of formol 5, acetic acid 5, and Müller's fluid 90, and stains in a variety of stains. See also Krause (*Virch. Arch.*, ccxviii, 1914, p. 107); Severingham (*Zeitschr. fur Zellf.*, xix, 1933, p. 635).

906. Thymus. The usual histological methods give good results. For hæmatological studies the Maximow Zenker fixative is recommended.

Dearth (*Amer. Journ. Anat.*, xli, 1928, p. 321) fixes in Carnoy's fluid (§ 90) and stains in hæmatoxylin followed by orange G or picro-fuchsin. See also Salkind (*Anat. Anz.*, xli, 1912, Nos. 6 and 7).

907. Adrenals. Chromaffin tissue, Wiesel (*Anat. Hefte.*, xix, 1902, p. 481) fixes one to four days in 5 per cent. $K_2Cr_2O_7$, 10 c.c.; 10 per cent. formol, 20 c.c.; distilled water, 20 c.c. Tissues are then placed in 5 per cent. $K_2Cr_2O_7$, for one or two days, washed one day in water and imbedded. Sections are stained twenty minutes in 1 per cent. toluidin blue or wasserblau, washed five minutes in water and stained twenty minutes in 1 per cent. safranin. They are then differentiated in 95 per cent. alcohol to a blue shade, then 95 per cent. alcohol, cleared in carbol xylol, xylol and mounted in Canada balsam.

In general chromaffin tissue is fixed and stained by those fixatives containing chrome or osmic acid by a simple reduction

due to adrenalin. Post chroming brings out the reaction in the former case.

Cramer (*Journ. Physiol., Proc. of Physiol. Soc.*, lii, 1918, p. 1) fixes the adrenals of rats and mice by suspending them in a wet gauze bag at 37° C. for one and a half hours in a closed tube containing osmic acid. The tissue is then transferred to 50 per cent. alcohol and is imbedded in wax. Sections may be mounted directly without further staining. Both cortical lipoids and adrenalin are blackened by the osmic acid, but the former staining is removed by turps. The author finds that the staining is better than with osmic acid bichromate solutions.

Ogata and Ogata (*Ziegl. Beitr.*, lxxi, 1923, p. 376), after fixation in Orths' formol (any formol bichromate fixative will do), stain twenty-four hours in Giemsa (10 drops to 10 c.c. distilled water). After washing this is differentiated in 0·25 per cent. acetic acid and brought through alcohol, xylol to balsam. A modification in the case of frozen sections permits their being dried and blotted and differentiated in acid free acetone, *i.e.* acetone which has been shaken up with calcium acetate in concentrated solution.

Hoare (*Amer. Journ. Anat.*, xlviii, 1931, p. 139) finds that the following mixture is the best fixative: $K_2Cr_2O_7$ $2\frac{1}{2}$ grm., $HgCl_2$ 5 grm., and water 100 c.c., to which neutral formol is added immediately before use in the proportion of 1 part formol to 9 of solution. Fixation is carried out for twenty-four hours, after which the pieces are put with various bichromate and osmic hardening mixtures for three to forty-two days. The original paper should be consulted in view of the large number of methods used.

Whitehead (*Journ. Path. and Bact.*, xxxv, 1932, p. 415) fixes in 5 per cent. $K_2Cr_2O_7$, to which an equal volume of 10 per cent. formol saline is added after two hours and fixation is carried out for twenty-four hours. He stains in hæmatoxylin and eosin.

908. For chromaffin glands in general, see Wistlocki (*Bull. Johns Hopkins Hospital*, xxxiii, 1922, p. 359), who employs very similar methods.

The intracellular lipoids may be studied in material fixed in osmic fixatives after paraffin imbedding. Post osmication is desirable. They may be stained in frozen sections by the usual fat stains.

Deansley (*Amer. Journ. Anat.*, xlix, 1931, p. 475) fixes in Flemming's chrome osmium mixture (§ 47) or uses Ciaccio's lipoid technique.

Whitehead (*Journ. Path. and Bact.*, xxxix, 1934, p. 443) uses the Schultz' cholesterol reaction (§ 662) for demonstrating this substance in the cortex; (*ibid.*, xii, 1935, p. 305) he uses the

Sudan IV. fat stain with frozen sections of material fixed in formol saline.

909. Pituitary. Numerous methods of differential staining have been developed for the anterior lobe cells. Good results are obtainable after most fixatives with Mallory's triple stain or Heidenhain's azan stain. See Bloom (*Anat. Rec.*, xlix, 1930, p. 363); Rasmussen (*Amer. Journ. Anat.*, xlvi, 1930, p. 461); Colin (*C.R. Soc. Biol.*, lxxxix, 1923, p. 1229); stains in mixtures of acid fuchsin, light green and methyl blue.

Cleveland and Wolfe (*Anat. Rec.*, li, 1932, p. 409) demonstrate four types of cell as follows. Fix in Regaud's fluid, changing each day, and transfer to 3 per cent. $K_2Cr_2O_7$, changing every twenty-four hours. Wash twenty-four hours and dehydrate slowly, cedar wood oil, xylol, and imbed at 60° C. Stain two to three sections in Ehrlich's hæmatoxylin for three minutes and rinse in distilled water. Blue in dilute lithium carbonate and transfer to 5 per cent. $K_2Cr_2O_7$, for three days, changing daily and keeping in the dark. Rinse in distilled water and stain twenty to thirty minutes in 5 per cent. erythrosin. Pass through two changes of distilled water and stain in 2 per cent. orange G in 1 per cent. phosphomolybdic acid for two or three minutes. Rinse and immerse in 1 per cent. anilin blue for thirty to sixty seconds. Rinse, pass through 95 per cent. alcohol and mount. Only Grübler's stains used. See also *ibid.*, v. 1931, p. 409, and *Ztschr. fur Zellf.*, xvii, 1933, p. 420.

Probably the best and simplest pituitary stain for routine work is that of Biggart (*Edinburgh Med. Journ.*, 1935, p. 42). Fix Zenker-formol eighteen to twenty-four hours. Stain thirty to thirty-five minutes at 50° C. in a mixture of equal parts of 0·5 per cent. aq. eosin yellow and 0·3 per cent. pyrrol blue (isamine blue). Differentiate in the following 5 per cent. sodium carbonate 10 parts, absolute alcohol 40 parts. Mount in Gurr's neutral mounting medium or neutral balsam. Acidophil cells red, basophils deep blue, chromophobe cells light blue.

Crook and Russel (*Journ. Path. and Bact.*, xl, 1935, p. 256) use the following method for differential cell counts after formol saline fixation. Sections are brought to water and are mordanted twelve to eighteen hours in 2·5 per cent. $K_2Cr_2O_7$ 95 parts, glacial acetic acid 5 parts, washed in running water for two minutes and placed in Lugol iodine for three minutes or more. They are decolourised in 95 per cent. alcohol for one hour or more and stained in 1 per cent. acid fuchsin for fifteen minutes. They are then washed in running water for thirty seconds to five minutes, rinsed in distilled water, and counterstained in Mallory's anilin blue mixture for twenty minutes. Lastly, they are washed in running tap water for two to five minutes and are differentiated in 95 per cent. alcohol for twenty seconds to five minutes. Absolute

alcohol, xylol, balsam. Before staining in acid fuchsin the nuclei may be stained in Ehrlich's hæmatoxylin, differentiated in acid alcohol. For combined methods giving cytological details as well as differential staining, see SEVERINGHAUS (*Anat. Rec.*, liii, 1932, p. 1) and (*ibid.*, lx, 1934, p. 43). See also MAURER and LEWIS (*Journ. Exper. Med.*, xxxvi, 1922, p. 141) and SPARK (*Journ. Lab. and Clin. Med.*, xx, 1935, p. 508) for further staining methods.

CHAPTER XXXV

BONE, TEETH, AND SKELETONS OF EMBRYOS *†

910. Bone, Non-decalcified. Ranvier (*Traité*, p. 297) has the following :

Bones should be plunged into water, without being allowed to dry, as soon as the surrounding soft parts have been removed, and should be divided into lengths with a saw whilst wet. The medulla should then be driven out from the central canal by means of a jet of water ; spongy bones should be treated as follows :

An epiphysis having been removed, together with a small portion of the diaphysis, a piece of caoutchouc tubing is fixed by a ligature on to the cut end of the diaphysis, and the free end of the piece of tubing adapted to a tap through which water flows under pressure ; they are then put to macerate for several months, the liquid being changed from time to time. As soon as all the soft parts are perfectly destroyed, the bones may be left to dry.

Thin sections may then be cut with a saw and prepared by rubbing down with pumice-stone. Compact pumice-stone should be taken and cut in the direction of its fibres. The surface should be moistened with water and the section of bone rubbed down on it with the fingers. When both sides of the sections have been rubbed smooth in this way, another pumice-stone may be taken, the section placed between the two, and the rubbing continued. As soon as the section is thin enough to be almost transparent it is polished by rubbing with water (with the fingers) on a Turkey hone or lithographic stone. Spongy bone should be soaked in gum and dried before rubbing down (but see Von Koch's copal process and Ehrenbaum's colophonium process).

Schaffer (*Zeit. wiss. Mik.* x, 1893, p. 171) grinds and polishes on stones of graduated fineness.

Röse (*Anat. Anz.*, vii, 1892, pp. 512–519) follows Koch's process. He penetrates first with a mixture of cedar oil and xylol, then with pure xylol, and imbeds in solution of Damar in chloroform or xylol. The method can be combined with Golgi's impregnation.

* For a detailed review of the whole subject see the paper of Schaffer in *Zeit. wiss. Mik.*, x, 1893, p. 167, or the article "Knochen und Zähne" in *Enzyk. mik. Technik.*

† J. T. C. and J. B. G.

Fanz (*Anat. Record*, xiv, 1918, p. 493) employs sand or carborundum paper of different grades of coarseness for grinding, using the back or smooth side of a piece of sandpaper for polishing the section. He recommends shellac in preference to balsam for attaching the section to the glass slip.

911. White (*Journ. Roy. Mic. Soc.*, 1891, p. 307) recommends the following: Sections of osseous or dental tissue should be cut or ground down moderately thin, and soaked in ether for twenty-four hours or more. They should then be put for two or three days into a thin solution of fuchsin in collodion, then into spirit to harden the collodion. After this they are ground down to the requisite thinness between two plates of old ground glass, with water and pumice powder, and mounted, *surface dry*, in stiff balsam or styrax, care being taken to use as little heat as possible. Lacunæ, canaliculi, and dentinal tubuli are found infiltrated by the coloured collodion.

Hanazawa (*Dental Cosmos*, lix, 1917, pp. 125 *et seq.*) gives a number of methods for staining ground and decalcified sections of dentine to demonstrate its minute structure.

Matschinsky (*Arch. mik. Anat.*, xxxix, 1892, p. 151, and xlvi, 1895, p. 290), after grinding, impregnates with nitrate of silver.

For similar method of Ruprecht, see *Zeit. wiss. Mik.*, xiii, 1896, p. 21, wherein see also quoted (p. 23) a method of Zimmermann.

Csokor (*Verh. anat. Ges.*, 1892, p. 270) describes a saw which will cut *fresh* bone to 120 μ; and Arndt (*Zeit. wiss. Mik.*, xviii, 1901, p. 146) a double saw which will also give very thin sections.

912. **Mounting.** To show lacunæ and canaliculi injected with air, take a section, or piece of very thin flat bone, quite dry. Place on a slide a small lump of solid balsam, and apply just enough heat to melt it. Do the same with a cover-glass, place the bone in the balsam, cover, and cool rapidly.

When thin ground sections of enamel are mounted in Canada balsam it is found often that they appear almost structureless. To demonstrate the enamel pattern of such sections they may be *etched* by immersion in 0·6 per cent. of hydrochloric acid in 70 per cent. alcohol, or in a weak aqueous solution of picric acid, and mounted in Camsal balsam or Euparal, media which, on account of their low index of refraction, will be found to disclose the structure of the enamel more easily.

913. **Ebner's Method for Bone Fibrillæ and Lamellæ.** The NaCl—HCl method recommended by Ebner is very useful when demonstration of the fibrillæ and lamellæ of bone tissue is desired. It is also applied for demonstration of the primitive rods. However, this method damages cell structures and nuclear staining.

Decalcification; solution; 100 c.c. of saturated sodium chloride is diluted with 100 c.c. of water and 1 c.c. (for teeth use 10 to 20 c.c.) of commercial hydrochloric acid is added. During the

decalcification process, 1 to 2 c.c. of HCl are added daily until the bones become flexible; they are then washed out for a few days in a saturated solution of sodium chloride. The solution soon becomes acid, but is continuously neutralised by the addition of traces of dilute ammonia. Rinsing out continues until the bones cease to lose acid.

Ebner's fluid acts very slowly, but gives good results. The thin well-washed sections are stained in gentian violet and differentiated with anilin oil and xylol (2 : 3). This process ensures the staining of the ground substance, fibrillæ of Ebner, and the Sharpey fibres.

914. Decalcification of Bones and Teeth, see Chapter XXVI., pp. 250–253.

915. Sections of Bones or Teeth showing the Soft Parts. A developing tooth with its epithelial enamel-organ, its mesodermal dentinal papilla, and its layers of partially calcified enamel and dentine, is made up of very delicate structures of different consistency and so is peculiarly liable to unequal shrinkage, with consequent distortion during the period of fixation and in the subsequent processes passed through in the preparation of sections. Further, post-mortem changes in the ameloblasts occur within a very few minutes after death, leading to a less precise behaviour to stains than is found in the case of cells which are fixed immediately after death.

For the examination of developing teeth *in situ*, jaws may be fixed in corrosive-formalin-acetic mixture, in Bouin's picro-formol, in Zenker's mixture or Helly's modification thereof, or in Sansom's modification of Carnoy's mixture (§ 90).

For the study of the micro-anatomy of the enamel-organ and the dentinal papilla, a young pup or a kitten, two or three days old, is killed, preferably by a blow on the head. The jaws are removed and the bone of the under-surface of the mandible pared away by a sharp scalpel until the bases of the tooth-germs are almost exposed. The muco-periosteum is grasped with a pair of forceps and stripped from the bone, when the tooth-germs will come away attached thereto.

Sansom's modification of Carnoy's mixture, employed *at blood-heat*, is particularly effective when the tooth-germs have been exposed in the manner outlined above, fixation therein being complete in from five to ten minutes. They are then passed through successive baths of alcohol of 30 per cent. and 50 per cent., each for fifteen minutes; 70 per cent., to which is added tincture of iodine, for four hours; 90 per cent. for thirty minutes; and into two changes of absolute alcohol, each for fifteen minutes or longer.

The tooth-germs are then transferred to a mixture of equal parts of absolute alcohol and carbon disulphide for one hour,

two changes of pure carbon disulphide, each of fifteen minutes, then for thirty minutes into carbon disulphide saturated with paraffin at 30° C., transferred to carbon disulphide saturated with paraffin at 42° C. for a like period, and finally into two baths of paraffin, in each half an hour. Imbed for cutting in pure paraffin.

By the employment of this method the amount of shrinkage in the tissues is extremely slight and the dentine does not become hardened, *so that the tooth-germs of the incisors may be cut without decalcification.* In the case of the canine and molar tooth-germs a short period of decalcification may be necessary, and for this purpose a rapid and delicate method lies in the employment of Zeigler's method (*Festschr. f. Küpffer*, 1899, p. 51), in which, by the use of a 5 per cent. solution of sulphurous acid, the insoluble tricalcium phosphate is changed into the readily soluble mono-calcium phosphate.

To demonstrate cytological detail no stain equals iron hæmatoxylin followed by a counterstain of picric-lichtgrun or of Rubin S in picrate of ammonia.

It cannot be too strongly emphasised that the precision of staining methods depends on the rapidity with which fixation of the tissues is effected after death.

For large jaws imbedding in celloidin, or, when serial sections are required, double imbedding in celloidin, parlodion or photoxylin and paraffin is recommended (§ 181).

Mummery (*Phil. Trans. B.*, ccviii, 1917, p. 258) deprecates the employment of paraffin for imbedding the tooth-germs of fishes, considering the heat employed to be very injurious to the delicate enamel organs, and advocates the use of the freezing method in obtaining sections. See carbon disulphide method above.

Nealey (*Amer. Mon. Mic. Journ.*, 1884, p. 142; *Journ. Roy. Mic. Soc.*, 1885, p. 348) says that perfectly *fresh* portions of bone or teeth may be ground with emery on a dentist's lathe, and good sections, with the soft parts *in situ*, obtained in half an hour.

Hopewell-Smith (*Journ. Brit. Dent. Ass.*, xi, 1890, p. 310; *Journ. Roy. Mic. Soc.*, 1890, p. 529) says that for preparing sections of teeth showing odontoblasts *in situ* the best plan is to take embryonic tissues. A lower jaw of an embryonic kitten or pup may be taken, and hardened in solution of Müller followed by alcohol, then cut with freezing microtome.

Weil (*Zeit. Mikr.*, 1888) fixes pieces of fresh teeth in sublimate, stains with borax carmine, brings them through alcohol into chloroform and chloroform balsam, and after hardening this by heat proceeds to grind as usual (§ 910).

916. For the study of the *vessels in teeth*, Lepkowsky (*Anat. Hefte*, viii, 1897, p. 568) injects with Berlin blue, hardens the teeth with a piece of the jaw for one or two days in 50 per cent.

formol, decalcifies in 10 per cent. nitric acid (eight to fourteen days, change frequently) and makes celloidin sections.

For decalcification of teeth, see also § 589 (Rousseau, Bödecker and Fleischmann). Bödecker finds Rousseau's process not applicable to *human* teeth : the acid *must* be added to the *fluid* celloidin.

For the study of the *lymphatics in the dental pulp*, Dewey and Noyes (*Dental Cosmos*, lix, 1917, pp. 436—44) first inject the blood-vessels with carmin-gelatin. Then 2 grm. of Prussian blue (oil colour in tubes) is stirred with 3 grm. of turpentine oil in a glass mortar for five minutes ; 15 grm. of sulphuric ether is added, and this fluid filtered through flannel or chamois skin. After the injection of this fluid the head is placed for twenty-four hours or longer in 20 per cent. formalin, and then the injected teeth are carefully removed and the pulps examined. Later it was found that more constant results were obtained when the injection of the blood-vessels followed that of the Prussian blue. Prussian blue injected directly into the pulps and trypan blue or lithium carmine injected intravenously or intraperitoneally were also employed. See §§ 515–553.

Wellings (*Proc. Sixth Internat. Dent. Cong.*, pp. 47 *et seq.*) demonstrated intra-vitam staining of dental and adjacent tissues by means of trypan blue (§ 748).

Mummery (*Phil. Trans. B.*, ccii, 1912), for the fixation of the *nerve-tissue* of the dental pulp, finds formalin to be preferable to all other fixing agents, employing 10 parts of the 40 per cent. commercial formalin to 90 parts of water.

Decalcification is effected by means of 33·3 per cent. formic acid. After thorough washing he leaves for twenty-four hours in a strong solution of dextrin (which he finds preferable to gum arabic), and sections are cut on the freezing microtome, by the employment of which he is able usually to obtain thinner sections than when paraffin is used for imbedding.

The sections are stained either by means of iron and tannin, iron hæmatoxylin (Benda), Congo red, Ranvier's modification of Löwet's gold chloride process, or by Cajal's method, where :—

1. Small pieces of the decalcified tooth, not more than 4 mm. thick, are placed in 50 c.c. of rectified spirit, to which 3 or 4 drops of ammonia may be added, and kept in this solution for from four to six hours.
2. Transfer to absolute alcohol for twenty-four hours.
3. Rinse with distilled water.
4. Place in a large quantity of 1·5 per cent. solution of silver nitrate, and keep in warm incubator at about 35° C. for five or six days.
5. Rinse in distilled water for a few seconds.

6. Place in the following solution for twenty-four hours :—

Hydroquinone	1 to 1·5 grm.
Distilled water	100 c.c.
Formol	5 to 10 c.c.
Rectified spirit	10 to 15 c.c.

7. Wash in water for some minutes.
8. Cut sections, and mount.

The presence of *nerve-end cells* in the dental pulp was demonstrated by Mummery (*Phil. Trans. B.*, ccix, 1920), by means of a modification of the gold method of Beckwith.

Teeth, immediately after extraction, are placed in a solution of formol and water or of formol and normal salt solution, preferably 4 per cent. of formol. This is, after a few days, changed to a 10 per cent. solution, and the teeth kept in this for at least a fortnight.

Decalcification is effected by means of a 33·3 per cent. solution of formic acid in distilled water, to which 5 per cent. of formol may be added. (Mummery states that neither he nor Dependorf has ever procured good nerve preparations of teeth which have been decalcified in the mineral acids.)

Wash in running water for twenty-four hours, then for a few minutes in distilled water.

The pieces are taken from the distilled water and suspended by threads in a large quantity of a weak solution of gold chloride (1 in 5000). Each piece should be suspended in at least 100 c.c. of the solution, in which it is left in the dark for from four days to one week, according to its size. On removal from the gold solution it is washed for a few minutes only in distilled water. Reduction is effected by placing the pieces in a 20 per cent. solution of caustic soda for four minutes, then rinsing in water and placing in a 10 per cent. solution of potassium carbonate for from half an hour to an hour. This is then drained off, and the pieces are placed in a 10 per cent. solution of potassium iodide for a short time—usually five to ten minutes. As soon as seen to darken, the pieces are removed from this solution to water, placed in gum for twelve hours, and sections cut on the freezing microtome.

After dehydration the sections are mounted in camsal (propylic) balsam.

917. Vivante (*Intern. Monatsschr. Anat. u. Phys.*, ix, 1892, p. 398) impregnates portions of frontal bone of four to six months' calves, which are not more than 3 to 4 millimetres thick, by Golgi's rapid bichromate and silver process. After impregnation the specimens should be decalcified in von Ebner's mixture (§ 913), well washed with water, and brought into solution of carbonate of soda, and finally imbedded in paraffin. For his quinolein blue method see *fourth edition*.

For Underwood's gold process for teeth, and for that of Lepkowski, see *third edition*, or *Anat. Anz.*, 1892, p. 294.

Law (*Proc. Roy. Soc. Med.*, i, 1908, p. 45) studies nerve-endings in teeth of mammals by treating paraffin sections of decalcified tissue with Bethe's molybdenum toluidin blue (details in *Journ. Roy. Micr. Soc.*, 1908, p. 518).

918. Van der Stricht (*Carnegie Instit. Embryol. Contrib.*, No. 21) fixes the isolated cochlea in a 5 per cent. aqueous solution of trichloracetic acid, or in Bouin's or Zenker's fluid, and stains, before imbedding, in borax carmine. The sections are afterwards stained in iron hæmatoxylin, Congo red and light green. He obtained the best results with the membrana tectoria by making one or two openings in the bony wall of the fresh cochlea and exposing the piece for fifteen minutes to the vapours from an aqueous solution of osmic acid or by submerging it in a 1 per cent. solution of the same for one hour. Afterwards fixation was completed by immersion in trichloracetic acid, Bouin's fluid or Zenker's fluid, and the series of sections therefrom stained as above. By this method some of the turns of the cochlea give very good preparations of the structure of the membrana tectoria. The mitochondria are also visible within osteoblasts, osteoclasts, connective-tissue cells, all epithelial cells, and the sensorial elements.

919. Mitochondria in odontoblasts and osteoblasts may be demonstrated by fixation in Regaud's fluid followed by staining in iron hæmatoxylin (§ 699), and the **Golgi apparatus** in these cells is well shown by the employment of Golgi's method, Aoyama's method, or of Da Fano's (§§ 719, 724), though a negative image of this cell-element is clearly shown when the tissues are fixed in Sansom's modification of Carnoy's mixture.

920. Bone, Decalcified (Flemming, *Zeit. wiss. Mik.*, 1886, p. 47). Sections of decalcified bone are soaked in water, dehydrated with alcohol under pressure, dried under pressure and mounted in hard balsam melted on the slide. They show the lacunar system injected with air as in non-decalcified sections.

921. Stains for Cartilage * and Decalcified Bone. See hereon Schaffer in *Zeit. wiss. Mik.*, v, 1888, p. 1; and *Enzyk. mik. Technik.*, art. "Knochen."

Kölliker (*Zeit. wiss. Zool.*, xliv, 1886, p. 662) treats sections of decalcified bone with concentrated acetic acid until they become transparent, and then puts for one quarter to one minute into a concentrated solution of indigo-carmine, washes and mounts in glycerin or balsam. The fibres of Sharpey appear *red*, the remaining bone substance blue.

Schaffer (*Zeit. wiss. Mik.*, v, 1888, p. 17) employed at one time a safranin method modified from Bouma (*Centralb. med. Wiss.*, 1883, p. 866), for which see *previous editions*. He now (*Encycl. mik. Tech.*, 1910, i, p. 762) stains sections for twenty-

* See under "Embryological Stains," § 811.

four hours in a bath of 20 c.c. of water with 1 drop of 1 per cent. solution of safranin (or thionin) and (apparently) mounts in balsam. The safranin stain will keep if the material is cartilage which has been fixed in picro-sublimate; otherwise it must be fixed with ammonium molybdate of 5 per cent. before dehydrating.

SCHMORL (*Centralb. allg. Path.*, x, 1899, p. 745) stains in a mixture of 2 c.c. concentrated solution of thionin in alcohol of 50 per cent. and 10 c.c. of water for ten minutes, rinses and puts into saturated aqueous picric acid for thirty to sixty seconds. Rinse and pass through graded alcohols into origanum oil or carbol-xylol and balsam. Matrix yellow, cells red, fat-cells violet. He also describes a more complicated method with thionin and phosphotungstic or phosphomolybdic acid.

MOLL (*Centralb. Physiol.*, xiii, 1899, p. 224) stains embryonic cartilage for six to twenty-four hours in orcein 0·5 gr., alcohol 40, water 20, hydrochloric acid 20 drops, and mounts in balsam. Matrix blue, nuclei red.

KALLIUS (*Anat. Hefte*, xxx, 1905, p. 9) stains first with borax carmine or alum carmine, then (sections) for ten minutes in saturated solution of thionin, and washes out with alcohol of 70 per cent. Said to be specific for embryonic cartilage.

VASTARINI-CRESI (*Att. Accad. med.-chir. Napoli*, 1907, p. 4) stains sections of embryonic cartilage with borax carmine, then with muchæmatein (alcoholic solution without acid), and then with Orange G in alcohol.

BAYERL'S *method for ossifying cartilage* (*Arch. mik. Anat.*, 1885, p. 35): Portions of ossified cartilage are decalcified as directed, § 250, cut in paraffin, stained in Merkel's carmine and indigo-carmine mixture, and mounted in balsam.

MAYER (*Grundzüge*, LEE and MAYER, 1910, p. 393) prefers to all these *resorcin fuchsin*, the precipitate being freed from iron chloride by washing before dissolving in the alcohol.

Aqueous solution of *benzoazurin* has been commended as a stain for ossifying cartilage by ZSCHOKKE, see *Zeit. wiss. Mik.*, x, 1893, p. 381.

A process of BAUMGARTEN'S has been given, § 427.

MOERNER (*Skandinavisches Arch. Physiol.*, i, 1889, p. 216; *Zeit. wiss. Mik.*, vi, 1889, p. 508) gives several stains for tracheal cartilage, chiefly as microchemical tests, for which see *third edition.*

See also a critique of these methods by WOLTERS in *Arch. mik. Anat.*, xxxvii, 1891, p. 492; and on the whole subject of cartilage see SCHIEFFERDECKER'S *Gewebelehre*, p. 331.

FUSARI (*Arch. Ital. Biol.*, xxv, 1896, p. 200) makes sections of fresh cartilage, puts them for twenty-four hours into 1 per cent. nitrate of silver, washes, dehydrates, and exposes to the light in balsam.

See also DISSE, *Anat. Anz.*, xxxv, 1909, p. 318, a stain for dentine (hæmalum followed by a mixture of Säurerubin and Orange G); and RETTERER and LELIÈVRE, *C. R. Soc. Biol.*, lxx, 1911, p. 630.

922. Cartilaginous Skeletons of embryos (VAN WIJHE, *Proc. K. Akad. Wetensch. Amsterdam*, 1902, p. 47) may be studied by

staining embryos for a week in a solution of 0·25 grm. methylen blue in 100 c.c. of 70 per cent. alcohol with 1 per cent. of hydrochloric acid. Wash out in alcohol with 1 per cent. of hydrochloric acid until no more colour comes away (about a week) and mount in balsam. The cartilage remains blue, all the other tissues being colourless.

Similarly, LUNDVALL (*Anat. Anz.*, xxv, 1904, p. 219, and xl, 1912, p. 639), using toluidin blue. Thionin blue also may be used.

Similarly also BAKAY (*Verh. Anat. Ges.*, 1902, p. 248), with Bismarck brown (the embryos having been previously treated with nitric acid of 3 per cent.).

For fish embryos, Professor E. S. Goodrich, of Oxford, informs us that thionin (Grübler) is excellent.

For the Spalteholz method of clearing such preparations see § 809.

923. Demonstration of Centres of Osteoblastic Activity by Trypan Blue (P. G. SHIPLEY and C. C. MACKLIN, *Anat. Record*, x, 1915—16). If an azo dye like trypan blue be administered to a very young animal, the bones are stained quickly and very intensely with vital colour. The dye is injected in a 1 per cent. solution into the peritoneal cavity (less preferably subcutaneously). The animal is killed forty-eight hours after staining, and the tissues are fixed by 10 per cent. neutral formalin injected through blood-vessels, followed by immersion in 10 per cent. formalin for twenty-four to forty-eight hours. Bones are washed thoroughly, hardened in ascending grades of alcohol, after which the soft parts are dissected away. Clear in benzol and then in oil of wintergreen. Study with dissecting microscope.

924. Potash Method for Osteoblastic Centres (SCHULTZE, *Grundriss d. Entwickl. d. Menschens*, 1897, and F. P. MALL, *Amer. Journ. Anat.*, v, No. 4, 1905–06).

Embryos of mammals after fixation in alcohol may be cleared, for the study of the ossification centres, by means of weak potash. For alcohol specimens Mall considers that Schultze's solution is too strong, and uses instead a 1 per cent. KOH solution for a few hours. With weak solutions the tissues of the smaller embryos remain firm, and, in the end, the specimen is transparent, with the bones held in place. After treatment with the potash, the embryo is placed in the following solution for days, or even months :—

Water	79 c.c.
Glycerin	20 c.c.
Potash	1 gm.

From time to time the embryo may be returned to a 3 per cent. solution of potash for a number of hours to hasten the clearing process ; then returned to the glycerin solution, which helps to hold the parts together. When properly cleared, upgrade gradually to pure glycerin, in which they may remain.

Mall (*op. cit.*) clears *formalin* embryos in 10 per cent. potash for about a month or longer. Formalin renders the connective tissues very tough, and this strong KOH solution is necessary. Refer also to § 809.

925. Dawson's Method of Staining the Skeleton of Cleared Specimens with Alizarin Red S (Sodium Alizarin Monosulphonate). Alden B. Dawson states (*in literis*) that the methods commonly used for obtaining a differential staining of developing bone in specimens already cleared or to be cleared (Lundvall, Spalteholz, Batson) have involved the staining of the entire specimen in a rather concentrated solution of alizarin, followed by differential decoloration. The alizarin is ordinarily made up as an alcoholic solution, usually in 95 per cent. alcohol. Such a solution stains the soft parts as well as the skeleton and must be followed by a decolourising fluid. Decoloration is accomplished, either by using an acid-alcohol, such as a ½ per cent. solution of sulphuric acid in 95 per cent. alcohol, or by allowing the specimen to stand in strong sunlight until the stain has been removed from the tissues surrounding the bones.

Decoloration by means of any acid solution is objectionable in a study of ossification on account of the danger of decalcifying minute ossification centres. Decolourisation in strong sunlight, while not open to the objection just raised for the acid method, also has some drawbacks. In continuous cloudy weather, it is obvious, no progress can be made and, in addition to this, many specimens prove very refractory to the sunlight method and the process of decoloration may in some cases take days, weeks or even months.

Dawson's new method has the advantage of introducing no factors of error due to possible decalcification and takes less time than bleaching in strong sunlight.

Legs of rats from one to thirty days of age have been prepared by this modified method. The animals are fixed *in toto* in 95 per cent. alcohol for from forty-eight to seventy-two hours, depending on the age of the animal. Prolonged fixation in the alcohol seems to render the tissue less liable to macerate in the potash solutions used later.

The tissue is then placed in a 1 per cent. solution of potassium hydroxide, Mall's (1902) modification of Schultze's (1897) method, for from twenty-four to seventy-two hours or until the bones are clearly visible through the muscle. The specimens are then placed in a dilute solution of alizarin and potash, 1 part alizarin to 10,000 parts of 1 per cent. potassium hydroxide. They are allowed to remain in this solution until the bones are stained the desired colour. If the dye is absorbed from the solution before the maximum intensity is obtained the specimen can be transferred to a fresh solution of stain. If clearing in the initial potash

solution has progressed to the proper stage, nothing but the bone will take up the stain. If the clearing has not been complete enough the muscles and other tissues take the stain almost as readily as the bone itself. Following the staining, the tissues are placed in Mall's solution, water 79 parts, glycerin 20 parts, and potash 1 part.

When properly cleared they are passed up through increasing concentrations of glycerin and stored in pure glycerin.

926. Dawson's Method for the Extraction of Fat from Embryos Prior to Clearing by the Potash Method. In specimens prepared by the potash method the fat in the superficial and muscular fasciæ is partially saponified and appears in the cleared material as opaque white masses which often prove a serious impediment to accurate observation.

This difficulty may be obviated by the extraction of the fat prior to beginning the treatment with KOH. Of the several common fat solvents tried, acetone was found to be most satisfactory. It acts quickly and does not injure the tissue or affect its clearing and staining qualities.

After the material has been fixed in 95 per cent. alcohol it is transferred directly to acetone and left there for several days, or longer, depending on the bulk of tissue being treated. Following this, the specimen is transferred directly back to 95 per cent. alcohol for twenty-four hours. After washing in 95 per cent. alcohol the clearing and staining are carried out as outlined above.

927. Osteoblasts *in Vitro*. J. C. Hill (*Arch. f. exper. Zellf.*, 1936) used the methods described in Chapters XXVIII and XXX.

CHAPTER XXXVI

TEGUMENTARY ORGANS OF METAZOA

928. Epithelium. Both for surface views and for sections good results are obtained by the *nitrate of silver* method, the *methylen blue* method, the *perchloride of iron and pyrogallol* method of the Hoggans, § 414, the *osmic acid and pyrogallol* process, § 413, and by *iron hæmatoxylin.*

For the purpose of separating the epidermis from the corium, LOEWY (*Arch. mik. Anat.*, xxxvii, 1891, p. 159) recommends macerating for twenty-four to forty-eight hours, at a temperature of about 40° C., in 6 per cent. pyroligneous acid. Acetic acid of $\frac{1}{3}$ per cent. (PHILIPPSON) is also good. MINOT (*Amer. Nat.*, xx, 1886, p. 575) macerates embryos for several days in 0·6 per cent. salt solution, MITROPHANOW (*Zeit. wiss. Mik.*, v, 1888, p. 573) for a quarter of an hour in 3 per cent. nitric acid, then one hour in one-third alcohol, and, if need be, twenty-four in stronger alcohol.

MAYER (*Lotos*, 2, xii, 1892) exposes the cornea or membrana nicitans of *Rana*, *Bufo*, and *Mus* for half a minute to the vapour of acetic acid, and then puts it into 0·5 per cent. salt solution.

For *ciliated epithelium* see the methods of Engelmann under "Mollusca."

929. Intercellular Bridges (and Canals), Prickle Cells. See IDE, in *La Cellule*, iv, 1888, p. 409, and v, 1889, p. 321; also KOLOSSOW, *Arch. mik. Anat.*, lii, 1898, p. 1. KOLOSSOW used an osmic-acid-tannin stain, § 413.

See also FLEMMING, *Anat. Hefte*, 1 Abth, vi, 1895, p. 1.

Besides maceration, impregnation may be useful; MITROPHANOW (*Arch. Anat. Phys., Phys. Abth.*, 1884, p. 191) has used gold chloride.

UNNA (*Monatsschr. prakt. Derm.*, xxxvii, 1903, p. 1) has described a highly complicated process with Wasserblau and orcein, see *Zeit. wiss. Mik.*, xxi, 1904, p. 68.

930. Plasma-fibrils of Epithelium. KROMAYER's process (*Arch. mik. Anat.*, xxxix, 1892, p. 141) is as follows: Sections are stained for five minutes in a mixture of equal volumes of anilin water (§ 694) and concentrated aqueous solution of methyl violet 6 B. They are well washed in water and treated with solution of iodine in iodide of potassium until they become blue-black (one to thirty seconds). They are again washed with water, dried

with blotting paper, and treated with a mixture of 1 volume of anilin to 2 volumes of xylol until sufficiently differentiated, when they are brought into pure xylol. Very thin sections will require more xylol in proportion to the anilin, viz. 1 : 3 or 1 : 4; thicker ones may require more anilin, viz., 3 : 5 or 3 : 3. Gentian or Krystallviolett will do instead of methyl violet, but not quite so well. See also EHRMANN and JADASSOHN, *Arch. Dermatol. u. Syphilis*, 1892, 1, p. 303; *Zeit. wiss. Mik.*, ix, 1893, p. 356; HERXHEIMER, *Arch. mik. Anat.*, liii, 1899, p. 510; and ROSENSTADT, *ibid.*, lxxv, 1910, p. 659 (takes the differentiating mixture much weaker in anilin).

UNNA (*Monatsschr. prakt. Derm.*, xix, 1894, p. 1 and pp. 277 *et seq.*; *Zeit. wiss. Mik.*, xii, 1, 1895, pp. 61, 63) has given a whole series of methods, from which the following are some extracts.

1. WASSERBLAU-ORCEIN. Stain sections for ten minutes in a neutral aqueous 1 per cent. solution of Wasserblau, rinse and stain for five or ten minutes in a neutral alcoholic 1 per cent. solution of Grübler's orcein. Dehydrate, clear, and mount in balsam. This may be varied as follows :—

(*a*) Ten minutes in the Wasserblau and thirty minutes or more in the orcein.

(*b*) Take for the second stain an *acid* solution of orcein.

(*c*) Stain for only one minute in the Wasserblau, but for thirty or more in the neutral orcein.

2. Stain for half an hour or more in a strong solution of hæmalum, rinse, stain for half a minute in a saturated aqueous solution of picric acid, and dehyrate for thirty seconds in alcohol containing 0·5 per cent. of picric acid.

3. Hæmalum for two hours, neutral orcein as above for ten to twenty minutes.

More recently UNNA advocates the process mentioned last section. See also under Pasini's method, § 812.

See also RANVIER, *Arch. Anat. Mikr.*, iii, 1899, p. 1.

931. Keratohyalin. The keratohyalin granules of the cells of the *stratum granulosum* are soluble in mineral acids, and can be digested in pepsin. They can be stained with picro-carmine, alum hæmatoxylin, van Gieson's mixture, or Unna's Wasserblau-orcein, last §. FICK (*Centralb. allg. Path.*, xiii, 1902, p. 987; *Zeit. wiss. Mik.*, xx, 1903, p. 222) stains sections of alcohol material for three to four minutes in concentrated aqueous solution of *Kresylechtviolett*, differentiates in alcohol, clears in xylol, and mounts in balsam.

See also UNNA, *Monatsschr. prakt. Derm.*, xx, 1895, p. 69; the article "Haut" in the *Encycl. mik. Technik.*; and UNNA and GOLODETZ, *Monatsschr. prakt. Derm.*, xlix, 1909, p. 95; LAFFONT, *Bibl. Anat.*, 1909, p. 209.

For *Trichohyalin*, see Gavezzeni, *Monatsschr. prakt. Derm.*, xlvii, 1908, p. 229.

932. Eleidin. To demonstrate the *stratum granulosum* and the eleidin granules Ranvier (*Arch. Anat. Micr.*, iii, 1899, p. 1) hardens with alcohol, stains with picro-carmine, and treats with lime-water. The cells swell and show up the granules, which do not change. See *loc. cit.*, other methods for the study of skin.

Buzzi (see *Encycl. mik. Technik.*, article "Haut") stains sections for a few minutes in a watch-glassful of water with 2 to 3 drops of 1 per cent. Congo red. Similarly Weidenreich, *Arch. mik. Anat.*, lvii, 1901, p. 583. Other authors recommend nigrosin, or Wasserblau, or orcein.

See also Joseph, "Dermatohist. Technik," Berlin, 1905, and Dreuw, *Med. Klink*, Berlin, 1907, Nos. 27 and 28.

For *Cholesterin* see Golodetz and Unna, *Monatsschr. prakt. Derm.*, xlvii, 1908, p. 1.

933. Horn, Hair, Nails and Feathers. The elements of hairs and nails may be isolated by prolonged maceration in 40 per cent. potash solution, or by heating with concentrated sulphuric acid. See also von Nathusius, *Zool. Anz.*, xv, 1892, p. 395.

Horny tissues stain well in safranin or gentian violet (Reinke, *Arch. f. mik. Anat.*, xxx, 1887, p. 183; Ernst, *ibid.*, lvii, 1896, p. 669; Rabl, *ibid.*, xlviii, 1896, p. 489).

Unna (*op. cit.* last section, p. 598) stains the tyrosin-bearing keratin in sections of skin for a few seconds or minutes in a mixture of 5 parts of Millon's reagent, 5 of water, and 1 of glycerin, treats shortly with nitric acid of 25 per cent., and mounts in balsam.

For Espinasse's method see § 177.

934. Skin-nerves and Nerve-endings. Impregnate with gold chloride. See Chapter XIX, especially § 398.

935. Tactile Corpuscles. See §§ 398–400. Gold methods are indicated. See also Ranvier, *Traité*, p. 919; Langerhans, *Arch. mik. Anat.*, 1873, p. 730; Kultschizky, *ibid.*, 1884, p. 358; and Smirnow, *Intern. Monatsschr. f. Anat.*, etc., x, 1893, p. 241, who recommends, besides the gold method of Löwit, the rapid bichromate of silver method of Golgi.

936. Corpuscles of Herbst and Corpuscles of Grandry. Dogiel (*Arch. Anat. u. Entwickel.*, 1891, p. 182) has used the methylen blue method. Four per cent. solution of methylen blue, warmed to 40° C., is injected into blood-vessels of the heads of ducks or geese; pieces of skin are removed from the beaks, sectioned in pith, and the sections brought on to slides and moistened with aqueous or vitreous humour from the animal and left for ten to thirty minutes exposed to the air, then brought into picrate of ammonia, and treated as described, § 382. Geberg (*Intern. Monatsschr. Anat.*, x, 1893, p. 205) made use of a method of

ARNSTEIN, according to which pieces of skin are put for twenty-four hours into lime-water, the horny layer removed, the pieces treated for five minutes with 0·25 per cent. gold chloride, reduced in water, and the precipitate that forms on them removed by putting into 0·25 per cent. cyanide of potassium and brushing.

NOWAK (*Anat. Anz.*, xxxvi, 1910, p. 217) takes UNNA'S Orceïn-wasserblau mixture (Wasserblau O.D., 1 part, orceïn 1, acetic acid 5, glycerin 20, alcohol 50, water 100) and adds to it 1 part more of orceïn. To 10 c.c. of this he adds at the moment of using 10 c.c. of 1 per cent. solution of eosin in alcohol of 80 per cent. and 3 c.c. of 1 per cent. solution of hydroquinone. Stain for five to ten minutes, rinse, stain for ten minutes in 1 per cent. aqueous solution of safranin, wash, treat for thirty minutes with 0·5 per cent. solution of bichromate of potash, dehydrate and mount.

Similarly DOGIEL, *Folia Neurobiol.*, iv, 1910, p. 218 (also employing Bielschowsky's neurofibril method).

937. Corpuscles of Meissner and of Krause (Cornea and Conjunctiva). DOGIEL (*Arch. f. mik. Anat.*, xxxvii, 1891, p. 602, and xliv, 1894, p. 15) employs the methylen blue method; for details see *previous editions.*

See also LONGWORTH'S methods, *Arch. mik. Anat.*, 1875, p. 655.

938. Similar Objects. Papillæ Foliatæ of the Rabbit, HERMANN, see *Zeit. wiss Mik.*, v, 1888, p. 524; ARNSTEIN, *ibid.*, xiii, 1897, p. 240. **Olfactive Organs of Vertebrates,** DOGIEL, *Arch. mik. Anat.*, 1887, p. 74. **Organs of a "Sixth Sense" in Amphibia,** MITROPHANOW, *Zeit. wiss. Mik.*, v, 1888, p. 513 (details as to staining with "Wasserblau," for which see also *Biol. Centralb.*, vii, 1887, p. 175). **Nerve-endings in Tongue of Frog,** FAJERSTAIN, *Arch. de Zool. expér. et gén.*, vii, 1889, p. 705. **Tongue of Rabbit,** VON LENHOSSEK, *Zeit. wiss. Mik.*, xi, 1894, p. 377 (Ramón y Cajal's double Golgi method).

939. Cornea. There are three chief methods—the methylen blue, the silver, and the gold method.

For the *methylen blue method* see particularly § 377.

Negative images of the corneal cells are easily obtained by the *dry silver method* (KLEIN). The conjunctival epithelium should be removed by brushing from a living cornea, and the corneal surface well rubbed with a piece of lunar caustic. After half an hour the cornea may be detached and examined in distilled water.

In order to obtain *positive* images of the fixed cells the simplest plan (RANVIER) is to macerate a cornea that has been prepared as above for two or three days in distilled water. There takes place a secondary impregnation.

The same result may be obtained by cauterising the cornea of a living animal as above, but allowing it to remain on the living animal for two or three days before dissecting it out, or by treating

a negatively impregnated cornea with weak salt solution or weak solution of hydrochloric acid (His).

But the best positive images are those furnished by *gold chloride*. Ranvier prefers his lemon-juice method. It is important that the cornea should *not remain too long in the gold solution*, or the nerves alone will be well impregnated.

Zawarsin (*Arch. mik. Anat.*, lxxiv., 1909, p. 116) removes the membrane of Descemet for study in the following manner. A cornea, fixed in sublimate, is dissected out and put for some hours into a mixture of alcohol and ether. The collodion of 4 per cent. is poured on to the inner surface, and after some time a layer of collodion with the membrane attached can be peeled off, and the collodion removed from the tissue by a mixture of alcohol and ether.

See also Rollett, in Stricker's *Handb.*, pp. 1102, 1115, or *previous editions;* Tartuferi, *Anat. Anz.*, v, 1890, p. 524, or *previous editions;* Ciaccio, *Arch. ital. Biol.*, iii, p. 75; and Renault, *C. R. Acad. Sc.*, 1890, p. 137.

940. Crystalline. Gerhardt (*Zeit. wiss. Mik.*, xiii, 1896, p. 306) hardens the lens for one or two days in 4 to 10 per cent. formalin; it is then easily dissociated with needles into its fibres.

Rabl (*Zeit. wiss. Zool.*, lxv, 1898, p. 272) fixes the enucleated eye for half an hour in his platinum chloride or picro-sublimate, §§ 80 and 75, divides it at the equator, and puts the anterior half back for twenty-four hours into the fixative.

For **Maceration** you may use sulphuric acid, § 575.

See also Robinski, *Zur Kenntnis d. Augenlinse*, Berlin, 1883.

CHAPTER XXXVII

MUSCLE AND TENDON (NERVE-ENDINGS)

STRIATED MUSCLE

941. Muscle-cells. For these and allied subjects see, *inter alia*, BEHRENS, KOSSEL, und SCHIEFFERDECKER, *Das Mikroskop*, etc., vol. ii, pp. 154–161; and SCHAFER, *Proc. Roy. Soc.*, xlix, 1891, p. 280.

Iron hæmatoxylin gives very fine images of striped muscle, and so does Mallory's phospho-tungstic.

For dissociation methods see §§ 554, 580.

To isolate the sarcolemma SOLGER (*Zeit. wiss. Mik.*, vi, 1889, p. 189) teases fresh muscles in saturated solution of ammonium carbonate.

942. Nerve-endings—the Methylen Blue Method. For BIEDERMANN's procedure for the muscles of *Astacus* see § 381 (see also *Zeit. wiss. Mik.*, vi, 1889, p. 65). After impregnating as there directed the carapace should be opened, and the muscles exposed to the air in a roomy moist chamber for from two to six hours.

For *Hydrophilus piceus*, BIEDERMANN proceeded by injecting 0·5 c.c. of methylen blue solution between the ultimate and penultimate abdominal rings, in the ventral furrow, and keeping the animals alive in water for three to four hours, then opened the thorax by two later incisions, and removed the muscles of the first pair of legs and exposed them to the air for three or four hours in a moist chamber, and finally examined in salt solution.

GERLACH (*Sitzb. Akad. Wiss. München*, 1889, ii, p. 125) injected frogs, either through the abdominal vein or through the aorta, with 4 to 5 c.c. of a 1 : 400 solution in 1 per cent. salt solution, and examined pieces of muscle in serum of the animal, afterwards fixing with picrate of ammonia and mounting in glycerin jelly.

The procedure of DOGIEL has been given, § 381.

943. Nerve-endings—the Gold Method. FISCHER (*Arch. mik. Anat.*, 1876, p. 365) used the method of LÖWIT.

BIEDERMANN (last section) recommends for *Astacus* a similar procedure, the preliminary treatment with formic acid being omitted, and the muscles being put for a couple of days into glycerin after reduction in the acid.

RANVIER (*Traité*, p. 813) finds that for the study of the motor

terminations of Vertebrates the best method is his lemon-juice process (§ 405).

See also the methods of Apáthy, §§ 407, 410.

944. Nerve-endings—the Silver Method. Ranvier employs it as follows (*ibid.*, p. 810); Portions of muscle (gastro-cnemius of frog) having been very carefully teased out in fresh serum, are treated for ten or twenty seconds with nitrate of silver solution of 2 to 3 per 1000, and exposed to bright light (direct sunlight is best) in distilled water. As soon as they have become black or brown they are brought into 1 per cent. acetic acid, where they remain until they have swollen up to their normal dimensions. They are then examined in a mixture of equal parts of glycerin and water.

This process gives *negative* images, the muscular substance being stained brown, and the nervous arborescence unstained. The gold process gives *positive* images, the nervous structures being stained dark violet.

945. Nerve-endings—the Bichromate of Silver Method. The *rapid* method of Golgi has been used by Ramón y Cajal for the terminations of nerves and tracheæ in the muscles of insects. See *Zeit. wiss. Mik.*, vii, 1890, p. 332, or *fourth edition*. A modification is used by Wunderer, *Arch. mik. Anat.*, lxxi, 1908, p. 523.

946. Muscle-spindles. See Cilimbaris, *Arch. mik. Anat.*, lxxv, 1910, p. 692. Principally *intra-vitam* methylen blue, by injection through the internal carotid. For elastic fibres Weigert's resorcin-fuchsin, followed by 1 per cent. orceïn acidified with HCl.

ELECTRIC ORGANS

947. Electric Organs. Ranvier (*Traité*, Chap. xviii), finds that osmic acid is the only reagent that will fix properly the terminal arborisations on the lamellæ. He injects a little 2 per cent. solution under the surface of the organ, removes a small portion of it after a few minutes, and puts it into a quantity of the same solution for twenty-four hours. The electric plates may then be teased out and examined in water, and will show the stag's horn ramifications; and the dissepiments between the columns will show the bouquets of Wagner. The terminal arborescence may be impregnated with silver. A portion of the surface of the organ is rubbed with lunar caustic until it appears opaque, then removed and the plates teased out in water. This gives negative images.

Or, electric plates, isolated by teasing after twenty-four hours in osmic acid as above, and kept for some days in one-third alcohol, are washed and placed on a slide with their ventral surface uppermost. They are then treated with a few drops of

0·5 per cent. solution of chloride of gold and potassium, and those which become violet are washed and mounted in glycerin. This gives positive images.

These may also be obtained by putting material fixed by osmic acid into 2 per cent. solution of bichromate of ammonia for a few weeks, then teasing, staining with alum hæmatoxylin, and mounting in damar.

Torpedo. BALLOWITZ (*Arch. mik. Anat.*, xlii, 1893, p. 460) gets the best results by the rapid Golgi impregnation.

An electric column, with about ½ to 1 cm. of tissue round it, is dissected out, and put for three to four days into the osmium bichromate mixture; then for one to three days into ¾ per cent. silver, cut without imbedding and mounted in xylol balsam. Impregnates all the important elements. See further, on the whole subject, BALLOWITZ, *Enzyk. mik. Techn.*, 1910, p. 298.

CAVALIÉ (*Bibl. Anat.*, xiii, 1904, p. 214) takes material fixed with osmic acid of 2 per cent. and impregnates it with gold by the method of NABIAS, and mounts in glycerin.

Raja. IWANZOFF (*Bull. Soc. Nat. Moscow*, ix, 1895, p. 74) fixes the organ in the tail of *Raja* with liquid of Flemming, stains with hæmacalcium and eosin, and makes paraffin sections.

BALLOWITZ (*Anat. Hefte*, 1 Abth., vii, 1897, p. 285) finds the method of Golgi excellent for this organ. He also makes sections after fixing in saturated solution of sublimate (in sea-water), or in liquid of Flemming, and examines them in water. Methylen blue may be used, *intra-vitam*. Gold is little good.

Gymnotus. BALLOWITZ (*Encycl. mik. Tecknik.*, p. 303) fixes with Flemming, and makes sections. He also commends impregnation with gold chloride, but not the Golgi method.

Malapterurus. BALLOWITZ (*ibid.*, p. 202) fixes with picro-sublimate, with Flemming, or with various mixtures of bichromate, sublimate, and formol, and uses gold chloride and Golgi impregnations. He macerates in liquid of Müller or saturated aqueous solution of picric acid.

TENDON

948. Tendons. RETTERER (*C. R. Soc. Biol.*, x, 1898, p. 580) fixes in equal parts of saturated solutions of sublimate and picric acid, puts for one to three days into saturated picric acid with 2 to 3 per cent. of sodium chloride, to remove the mucin, and imbeds in paraffin.

949. Union of Muscle and Tendon. For this see RETTERER and LELIÈVRE, *C. R. Soc. Biol.*, 1911, No. 12 (orceïn for twenty-four hours, followed by iron hæmatoxylin); and SCHULTZE (*Verh. phys. med. Ges. Würzburg*, 1911, p. 33) treats for a day or two with a mixture of equal parts of 2 per cent. bichromate of potash

and alcohol, in the dark, then for two days with 0·5 per cent. solution of hæmatoxylin in alcohol of 70 per cent., then with Van Gieson's picro-säurefuchsin).

950. Corpuscles of Golgi (Ranvier, *Traité*, p. 929). Take the tendon of the anterior and superior insertion of the gemini muscles of the rabbit. Treat it by the formic acid and gold method (§ 404), and after reduction scrape with a scalpel, in order to remove the muscle-fibres that mask the musculo-tendinous organs.

Marchi's methods for the tendons of the motores bulbi oculi (*Archivio per le Scienze Mediche*, v, No. 15). The enucleated eyes, together with their muscles, were put for not less than three days into 2 per cent. bichromate of potash. The muscles and tendons were then carefully dissected out, stained with gold chloride and osmic acid (Golgi's method), and by the method of Manfredi, § 407. Mount in glycerin. The methods only succeed completely during fine, sunny weather.

Ruffini (*Atti R. Acc. Lincei Roma Tend.* [5], i, 1892, p. 442) recommends the method of Fischer.

Ciaccio (*Mem. R. Acc. Sci. Bologna* [4], t. x, 1890, p. 301) puts tendons of Amphibia into 0·1 per cent. hydrochloric acid or 0·2 per cent. acetic acid until transparent; then for five minutes into a mixture of 0·1 per cent. gold chloride and 0·1 per cent. potassium chloride; then back into the acetic acid, for a day in the dark, and for two or three hours more in the sunlight. When they have become somewhat violet they are put for a day into 0·1 per cent. osmic acid, and finally mounted in glycerin acidulated with 0·5 per cent. of acetic or formic acid.

Dogiel (*Arch. mik. Anat.*, lxvii, 1906, p. 638) stretches tendons of eye-muscles on cardboard with hedgehog spines, puts for four or five days into nitrate of silver of 1 to 2 per cent., reduces for a day in pyrogallic acid with formol, and imbeds in celloidin.

SMOOTH MUSCLE

951. Tests for Smooth Muscle. Picro-säurefuchsin, § 280, stains muscle yellow, connective tissue red.

Picro-nigrosin, § 353, stains muscle yellowish, connective tissue blue.

Unna (*Encycl. mik. Technik.*, article "Kollagen") stains for twenty-four hours in orceïn 1 part, Wasserblau 0·25, alcohol 60, glycerin 10, water 30, which gives muscle in a mixed tone, collagen blue, elastin reddish. See also a complicated process with methylen blue in *Monatssch. prakt. Dermatol.*, xix, 1894, p. 533, and another with orceïn, hæmateïn, säurefuchsin and picric acid.

Retterer (*C. R. Soc. Biol.*, 1887, p. 645) fixes in 10 volumes

of alcohol with 1 of formic acid, washes well and stains in alum carmine. Muscle red, connective tissue unstained.

952. General Structure. WERNER (*Hist. d. glatten Musculatur*, Dorpat, 1894, p. 22) fixes stretched intestine or bladder in Flemming, washes well and stains in Heidenhain's chrome hæmatoxylin, § 305. For demonstrating intercellular spaces, fresh intestine is put for twenty-four hours into oil, at 37° C., then for twelve hours into Flemming, and for four to six into chromo-acetic acid.

953. Isolation of Fibres. GAGE's methods, see §§ 578, 560.

MOBIUS, muscle at *Cardium*, see § 569.

BALLOWITZ, muscle of Cephalopoda, see *Arch. mik. Anat.*, xxxix, 1892, p. 291.

SCHULTZ (*Arch. Anat. Phys., Phys. Abth.*, 1895–6, p. 521) puts muscle of Vertebrates for twenty-four hours into 10 per cent. nitric acid, rinses with water, and brings pieces for six to eight days (in the dark at first) into a mixture of equal parts of $\frac{1}{20}$ per cent. osmic acid and $\frac{1}{5}$ per cent. acetic acid, teases and mounts in glycerin.

For smooth muscle of Vermes, see APÁTHY, *Zeit. f. wiss. Mik.*, x, 1893, pp. 36, 319, and § 572, *ante*.

954. Iris. DOGIEL (*Arch. mik. Anat.*, 1886, p. 403) puts the anterior half of an enucleated eye for some days into a mixture of 2 parts of one-third alcohol and 1 part 0·5 per cent. acetic acid. The iris can then be isolated, and split from the edge into an interior and posterior plate, and these stained according to the usual methods.

See also KOGANEI, *Arch. mik. Anat.*, 1885, p. 1; CANFIELD, *ibid.*, 1886, p. 121; and DOSTOIEWSKY, *ibid.*, p. 91.

955. Bladder of Frog, Innervation of (WOLFF, *Arch. f. mik. Anat.*, 1881, p. 362). A frog is killed and a solution of gold chloride of 1 : 20,000 injected into the bladder through the anus. (If the injection flows out on removal of the syringe, tie the frog's thighs together.) Now open the frog, ligature the intestine above the bladder, and cut away the abdomen so as to have in one piece bladder, rectum and hind legs. Put this into gold solution of 1 : 2000 for four hours; the bladder is then excised, slit open and pinned (with hedgehog spines) on to a cork (outside downwards). Place it under running water until all the epithelium is washed away. Use a camel's-hair brush if necessary. Put for twenty-four hours into gold solution of 1 : 6000. Wash in pure water, and put away in the dark "for some time" in acidulated water, and finally reduce in fresh water in daylight.

RANVIER (*Traité*, p. 854) recommends his two gold processes, the liquids being injected as above.

GRÜNSTEIN (*Arch. mik. Anat.*, 1899, p. 1) injects 1 per cent.

methylen blue in normal salt solution through the *vena abdominalis*, and after twenty to thirty minutes excises the bladder and exposes to the air. Fix the stain with picrate of ammonia and mount in glycerin with the same (§ 382).

CHAPTER XXXVIII

CONNECTIVE AND ELASTIC TISSUES

CONNECTIVE TISSUE

956. General Stains for Connective Tissue. Connective tissue, elastic tissue, and smooth muscle are all *normally* acidophilous. Collagen, the distinctive element of connective tissue, absolutely requires "acid" dyes for the production of a permanent stain, whilst elastic tissue and muscle will also fix "basic" dyes. Collagen has a special affinity for Säurefuchsin and Wasserblau. Elastin has a strong affinity for acid orceïn, whilst muscle has no special affinity for either, but stains energetically with picric acid.

Picro-säurefuchsin is much used and very convenient as a general differentiating stain, but not to be recommended for cytological detail. See SCHAFFER, *Zeit. wiss. Zool.*, lxxx, 1905, p. 176.

E. and T. SAVINI recommend BENDA's Picro-Säurefuchsin, § 326.

EHRLICH-BIONDI mixture gives connective tissue red, but smooth muscle redder still.

UNNA's *Wasserblau-orceïn* for distinguishing connective tissue and muscle has been given, § 930. It works after all fixatives. Stain long, and dehydrate preferably with acid alcohol.

FREEBORN (*Amer. Mon. Mic. Journ.*, 1888, p. 231) recommends (for sections) *picro-nigrosin*, made by mixing 5 c.c. of 1 per cent. aqueous solution of nigrosin, with 45 c.c. of aqueous solution of picric acid. Stain for three to five minutes, wash with water, and mount in balsam. Connective tissue blue, nuclei blackish, the rest yellowish.

RAMÓN Y CAJAL's *picro-indigo-carmine* gives connective-tissue fibres dark blue, with red nuclei.

S. MAYER (*Sitzb. k. Akad. Wiss.*, lxxxv, 1882, p. 69) recommends for staining *fresh* tissue Violet, B, § 357. Elastic fibres and smooth muscle also stain, but of different tints.

DUBREUIL (*C. R. Ass. Anat.*, vi Sess., 1904, p. 62) uses a mixture of 28 volumes 1 per cent. picric acid and 2 volumes 1 per cent. methyl blue—with a previous stain with carmalum or safranin.

For RANVIER's method of artificial œdemata for the study of areolar tissue, see his *Traité*, p. 329.

957. UNNA's **Orceïn Method.** (*Encycl. mik. Technik.*, 1910, p. 250). Sections are stained for ten minutes in Grübler's poly-

chrome methylen blue. They are then washed with water, mopped up, and brought for fifteen minutes into a *neutral* 1 per cent. solution of orceïn in absolute alcohol, rinsed in pure alcohol, cleared in bergamot oil, and mounted. Collagenous ground-substance dark red, muscle bluish, elastic fibres *sometimes* dark red. Material may be fixed in almost any way except with nitric or picric acid, formol, or liquids of Müller and Hermann.

958. UNNA's **Methylen blue + Saurefuchsin** (UNNA, in *Enzyk. mik. Technik.*, 1910, p. 247). Stain for two to five minutes in polychrome methylen blue solution (Grübler). Wash and stain for ten to fifteen minutes in "(0·5 per cent.) Säurefuchsin + (33 per cent.) tannin-mixture (Grübler)." Water, alcohol, essence, balsam. Collagen, protoplasm, and muscle red, nuclei and keratin blue. On Flemming material, elastin blue. Liquids of Hermann and Erlicki, formol and copper fixatives incompatible.

959. UNNA's **Safranin + Wasserblau** (*ibid.*). Ten minutes in 1 per cent. safranin. Wash. Ten to fifteen minutes in "(1 per cent.) Wasserblau + (33 per cent.) tannin mixture." Wash. Stains in opposite colours to the last. Formol and liquid of Hermann contra-indicated for fixing.

960. **Flemming's Orange Method** is said to give a very sharp differentiation of *developing fibrils*.

961. MALLORY (*Zeit. wiss. Mik.*, xviii, 1901, p. 175) stains sections of sublimate or Zenker material for a few minutes in Säurefuchsin of 0·1 per cent. mordants for a few minutes in 1 per cent. phosphomolybdic acid and stains for two to twenty minutes in anilin blue 0·5 grm., Orange G 2, oxalic acid 2, and water 100. His phosphotungstic hæmatoxylin stains connective tissue sharply, but does not differentiate it sufficiently from elastic tissue and muscle.

962. For the complicated procedure of HORNOWSKI see *ibid.*, xxvi, 1909, p. 138.

963. For DELAMARE's mixture or orceïn, hæmatoxylin, Säurefuchsin and picric acid see *Verh. Anat. Ges.*, xix, 1905, p. 227.

964. MASSON (*C. R. Soc. Biol.*, lxx, 1911, p. 573), stains first in hæmalum, then in eosin, and then for a few minutes in 1 per cent. solution of saffron in tap-water (made by boiling). Connective tissue, bone, and cartilage, yellow.

965. **Benecke's** stain for fibrils (*Verh. Anat. Ges.*, vii, 1893, p. 165) is essentially that of KROMAYER, § 930.

966. **Bielschowsky's** SILVER METHOD (*post, under* "*Neurofibrils*") has been used for connective-tissue fibrils. SNESSAREW (*Anat. Anz.*, xxxvi, 1910, p. 401) employs it as follows: Tissue is hardened in neutral formol and sectioned with a freezing microtome. The sections are put for at least four days into *iron alum* of 2·5 to 10 per cent., changed daily. They are then silvered for thirty-six to forty-eight hours in nitrate of silver of 10 per cent., then treated with the oxide bath and reduced in formol of 20 per cent. Collagen fibres grey, but fine

connective networks black, nerve fibres unstained or only weakly stained.

Perdrau (*Journ. Pathol. Bact.*, xxiv, 1921) has worked out a modification of Bielschowsky which appears to be particularly suitable for the study of the connective tissue in nervous organs. He washes pieces and sections as in Da Fano's modifications, but without having recourse to re-distilled water. He then places sections for about ten minutes in 0·25 per cent. potassium permanganate, washes, and treats them as by Pal's modification of Weigert's myelin stain (see § 1058). After another wash in distilled water, he transfers sections into 2 per cent. silver nitrate, and continues as in Da Fano's Mod. 1. Nerve-cells, nerve-fibres, neuroglia, etc., *unstained;* connective tissue and elastic fibres stained in various shades of purple-grey to black. Refer to § 1055.

967. H. Wilder's **Silver Impregnation Method for Reticulum Fibres** (*Amer. Journ. Path.*, xi, 1935). Fix in 10 per cent. formol, acetic Zenker or formol Zenker. Mount paraffin sections with egg albumen, and bring down to water. Place in 1 per cent. pot. permanganate for half to one minute. Wash in distilled water. Place in 5 per cent. oxalic acid for one minute. Wash well in distilled water. Place in 1 per cent. uranium nitrate for five to ten seconds. Rinse rapidly in distilled water.

Place in the following solution for one minute :—

To 5 c.c. of a 10·2 per cent. silver nitrate solution, add ammonia drop by drop until the precipitate disappears. Add 5 c.c. of a 3·1 per cent. solution of NaOH, and just dissolve the resulting precipitate with a few drops of ammonia. Make up to 50 c.c. with distilled water.

Dip quickly in 90 per cent. alcohol.

Reduce for one minute in the following solution :—

Distilled water	50 c.c.
40 per cent. neutral formalin . .	0·5 ,,
1 per cent. uranium nitrate . . .	1·5 ,,

Wash in distilled water. Tone in 0·2 per cent. gold chloride for half to one minute. Rinse in distilled water. Place in 5 per cent. hypo for one to two minutes. Wash well in water. This is a remarkably good method recommended by K. C. Richardson.

See also Maresch, *Zeit. wiss. Mik.*, xxiii, 1906, p. 356; Studnicka, *ibid.*, p. 416; Zimmermann, *ibid.*, xxv, 1908, p. 10; Levi, *Monit. zool. Ital.*, 1908, p. 290; Heinrich, *Arch. mik. Anat.*, lxxiv, 1909, p. 786 (dentine); Insabato, *Arch. Ital. Anat. Emb.*, viii, 1909, p. 375 (silvers Flemming material); Athanasiu and Dragoiu, *C. R. Acad. Sci.*, cli, 1910, p. 551 (Ramón y Cajal's silver process, with alcohol fixation).

ELASTIC TISSUE

968. Elastic Tissue, Generalities. Elastic fibres have a great affinity for osmium, staining with much more rapidity than most other tissue elements. They are not changed by caustic soda or potash. They are *normally* acidophilous, but are easily rendered *artificially* basophilous by means of chromic acid or other mordants, and then stain with great energy with basic dyes. Hence a group of stains of which those of Lustgarten and Martinotti are types. They have a natural affinity for orceïn, whence stains of the Taenzer-Unna type.

For a review of the older methods of Balzer, Unna, Lustgarten, and Herxheimer, see the paper by G. Martinotti, in *Zeit. wiss. Mik.*, iv, 1887, p. 31; also *Enzyk. mik. Technik.*, art. "Elastin."

969. Victoria Blue (Lustgarten). See § 375.

970. Safranin (G. Martinotti, *loc. cit.*, § 968). Fix in a chromic liquid, wash, stain for forty-eight hours in strong (5 per cent. Pfitzner's) solution of safranin, wash, dehydrate, clear, and mount in balsam. Elastic fibres black.

The staining will be performed quicker if it be done at the temperature of an incubating stove (Griesbach, *ibid.*, iv, 1887, p. 442). See also Ferria (*ibid.*, v, 1888, p. 342).

See also Mibelli, *Mon. Zool. Italiano*, 1, p. 17, or *Zeit. wiss. Mik.*, vii, 1890, p. 225 (the report in *Journ. Roy. Mic. Soc.*, 1890, p. 803, is vitiated by a misprint). Other basic dyes have been recommended.

971. Kresofuchsin (Rothig, see § 375).

972. Orceïn. This method is due to Taenzer, and as modified by Unna is known as the Taenzer-Unna method, see *third edition*, or *Monatssch. prakt. Dermatol.*, xii, 1891, p. 394.

Unna's **Modified Orceïn Method** (*Monatssch. prakt. Dermatol.*, xix, 1894, p. 397; *Zeit. wiss. Mik.*, xii, 1895, p. 240). Grübler's orceïn 1 part, hydrochloric acid 1 part, absolute alcohol 100 parts. Stain sections for thirty to sixty minutes, or for ten to fifteen at 30° C., rinse in alcohol, clear, and mount. Elastin dark brown, collagen light brown.

See also *Merk. Sitz. Akad. Wiss. Wien*, cviii, 1899, p. 335; Pranter, *ibid.*, xix, 1903, p. 361 (he takes 2 per cent. of nitric acid instead of the hydrochloric, and stains six to twenty-four hours); Wolff, *ibid.*, p. 488; the article "Elastin" in *Enzyk. mik. Technik.*; and E. and T. Savini, *Zeit wiss. Mik.*, xxvi, 1909, p. 34.

973. Weigert's **Resorcin-Fuchsin Method** (*Centralb. allg. Path.*, ix, 1898, p. 290). One per cent. of basic fuchsin and 2 per cent. of resorcin (or of carbolic acid) are dissolved in water. Two hundred cubic centimetres of the solution are raised to boiling-point in a capsule, and 25 c.c. of *Liquor ferri sesquichlorati* P. G. are added, and the whole is boiled, with stirring, for two to five

minutes more. A precipitate is formed. After cooling the liquid is filtered, and the precipitate which remains on the filter is brought back into the capsule, and there boiled with 200 c.c. of 94 per cent. alcohol. Allow to cool, filter, make up the filtrate to 200 c.c. with alcohol, and add 4 c.c. of hydrochloric acid.

WOLFRUM (*Zeit. wiss. Mik.*, xxv, 1908, p. 219) adds 10 to 15 per cent. of acetone to the mixture.

Stain sections (of material fixed in any way) for twenty minutes to an hour, wash with alcohol, clear with xylol (not with an essence). Elastic fibres dark blue on a light ground.

Crystal Violet for Weigert's Elastic Tissue Stain (SHERIDAN, *Journ. Tech. Meth. and Bull. Inter. Assn. Med. Museums,* 1929). Crystal violet 1 grm., resorcin 2 grm., distilled water 100 c.c. Dissolve by boiling for a few minutes, stirring constantly. Add to boiling mixture 30 c.c. of a 30 per cent. aqueous solution of ferric chloride. Continue boiling, stirring constantly, for several minutes or until precipitation ceases. Collect precipitate on the filter and wash with 50 c.c. of distilled water. Dissolve in 100 c.c. of absolute alcohol by boiling in a porcelain capsule on a sand-bath. Cool, filter and add 2 c.c. HCl. Stain sections for one or two hours and differentiate in absolute alcohol till elastic fibres alone remain.

MINERVINI (*Zeit. wiss. Mik.*, xviii, 1901, p. 161) gives a variant with safranin instead of fuchsin.

See also PRANTER, *ibid.*, xix, 1903, p. 361 ; B. FISCHER, *Virchow's Arch.*, clxx, 1902, p. 285, or *Zeit. wiss. Mik.*, xx, 1903, p. 40 (chemistry of the dyes obtained by these processes, which he calls " Fuchselin," " Safranelin," etc.) ; HART, *Centralb. allg. Path.*, xix, 1908, p. 1 ; and CILIMBARIS, *Arch. mik. Anat.*, lxxv, 1910, p. 708.

974. Hæmatoxylin Methods. HARRIS (*Zeit. wiss. Mik.*, xviii, 1902, p. 290) makes an " Elasthæmatein " as follows : Hæmatoxylin 0·2 grm., aluminium chloride 0·1 grm., alcohol of 50 per cent. 100 c.c., boil and add mercuric oxide 0·6 grm., filter and add 1 drop of HCl. Keep for some weeks. Stain for five or ten minutes, put into alcohol with 1 per cent. of nitric acid for one minute, then pure alcohol.

See also DE WIT, *Anat. Rec.*, i, 1897, p. 74 ; DUERCK, *Arch. Path. Anat.*, clxxxix, 1907, p. 62 ; VERHOEFF, *Journ. Amer. Med. Assoc.*, 1908, No. 11.

MALLORY's phosphotungstic hæmatoxylin is good, but not specific.

For a hæmatoxylin and eosin stain for connective tissues see KRÜGER (*Zeit. f. w. Mikr.*, xxxi, or *Journ. R. Micr. Soc.*, 1914).

975. Other Methods for Elastic Tissue. For the elastic tissue of the skin see PASSARGE and KRÖSING, *Derm. Stud.*, xviii, 1894.

See also for staining and dissociation AGABABOW, *Arch. mik. Anat* , l, 1897, pp. 566 *et seq.*

For C. MARTINOTTI's silver impregnation see *Zeit. wiss. Mik.*, v, 1888, p. 521, or *Arch. Ital. Biol.*, xi, 1889, p. 257.

SCHUMACHER (*Arch. mik. Anat.*, lv, 1899, p. 151) has had good results (for the spleen) with picro-nigrosin.

976. DREW-MURRAY van Gieson-Nile Blue Method for Connective Tissues (and Bacteria). Fix in formol-salt solution.

Prepare paraffin (or frozen) sections. Stain one to three minutes in van Gieson's picric acid-acid fuchsin solution. Wash in aq. dest.; treat in 2 per cent. Nile blue sulphate solution in aq. dest. for from two to twenty-four hours. Wash in changes of aq. dest. till the latter is tinted pale blue. Stain again in van Gieson one to five minutes. Wash in aq. dest. till wash-water is pale yellow. Dehydrate rapidly with absolute alcohol from drop bottle. Clear quickly in xylol (not more than a minute). Differentiate in clove oil from five minutes to several hours (the longer period is for frozen sections). Wash in xylol—Canada balsam.

In successful preparations nuclear chromatin a saturated transparent blue, mast cell granules nearly black, collagen red, keratin and erythrocytes orange yellow (if bacteria are present they stain blue). (*Report of Imper. Cancer Research Fund,* 1919.)

CHAPTER XXXIX *

NERVOUS SYSTEM—GENERAL METHODS

977. Introduction. The methods for the microscopical investigation of the nervous systems can be subdivided into two groups which are spoken of as the cytological and anatomical methods respectively. Those belonging to the first group are chiefly meant for the study of the intimate structure of nerve-cells, nerve-fibres and their supporting tissues; those forming the second group are more particularly suitable for investigating the morphology of nerve-cells and their connections with one another and with the nerve-fibres, as well as the architectural arrangement of both nerve-cells and nerve-fibres in the various regions of the central nervous system. This subdivision has mainly a descriptive purpose, because methods which were originally proposed for the study of the nerve-cell structure were afterwards found useful for investigating the distribution of nerve-cells in the grey substance, while on the other hand slight modifications of anatomical methods have led to the discovery of important cytological details.

The methods for the study of the nerve-tissue in peripheral organs having been described in the chapters on "Methylen Blue," "Impregnation Methods," "Tegumentary Organs" and "Muscle and Tendon," the following chapters are meant chiefly for the description of methods for the investigation of the central nervous system.

FIXATION

978. Fixation by Injection. Fixation, in the proper sense of the word, is of course out of the question for human material. But in the case of animals it is possible to inject fixing fluids into their nervous centres when still in an almost living state. The practice ensures a very rapid penetration into and even distribution within the tissues of the fixing agents, and has the capital advantage of greatly helping to prevent distortion of the nerve-tissues during their subsequent treatment. As in most instances the practice does not meet with special difficulties, it should be adopted as far as possible also in the case of man, but particularly for a preliminary fixation and hardening of the very soft cerebral mass of young individuals, which is particularly liable to injury and distortion in the process of removing it from the brain case.

The choice of the fluid to be injected depends upon the object

* Revised by J. G. G. and R. O. S.

in view and the subsequent treatment to which the tissues are to be submitted. In the case of animals it is a good practice to warm the fixing fluid to body-temperature before injecting it, and, whenever possible, to wash out the blood by first injecting physiological solution as suggested by Mann. The injection can be carried out through the carotids if the fixation is to be limited to the encephalon, and through the aorta if it is desired to fix the spinal cord too. The above applies to higher vertebrates and particularly to mammals; in the case of lower vertebrates, fixation by injection has not, as a rule, the same importance, and one must have recourse to special methods.

See on this subject Golgi, *op. cit.*, § 1027; Gerota, § 983; De Quervain, *Virchow's Arch.*, cxxxiii, 1893, p. 515; Mann, *Ztschr. wiss. Mikr.*, xi, 1894, p. 482; Strong, *Anat. Anz.*, xi, 1896, p. 655; *Journ. Comp. Neurol.*, xiii, 1903, p. 291; McFarland, *Journ. App. Micr.*, ii, 1899, p. 541; or *Ztschr. wiss. Mikr.*, xvii, 1900, pp. 39, 40.

979. Hardening by Freezing. This phrase has often given rise to confusion and should, therefore, be clearly understood. One can harden by freezing either fresh tissues, or material already fixed and consequently also a little hardened. In the first instance small pieces of fresh tissue, immediately after removal and without any previous treatment, are hardened on a freezing microtome. The sections are generally floated on to water, and immediately afterwards treated for a minute on the slide with a 0·25 per cent. solution of osmic acid; or otherwise treated according to the object of one's investigation. Chilling the knife after the method of Schutz-Brauns (*Virchow's Arch.*, 1929, cclxxiii, p. 1) is particularly useful. Since Brodmann (*Journ. Psychol. u. Neurol.*, ii, 1903–04, p. 211) has shown that formalin material can be used even for investigations by polarised light, section of unfixed tissue is rarely needed. (See also p. 895.)

The hardening by freezing of already fixed material may be also attended with some difficulty, but this will be easily overcome if pieces are relatively small, the fixing agent properly washed away, and one has, eventually, recourse to one or other of the processes described in § 197. Nervous tissue well fixed in formalin is very suitable for frozen sectioning. Care must be taken that the material is not too hard when cut. For a description of the freezing technique refer to Chapter XII.

The hardening and section cutting by the freezing method of very large pieces require special apparatus and special methods, for which see Nageotte, *C. R. Soc. Biol.*, lxvii, 1909, p. 542. The large freezing box made for Reichert's sledge microtome is very suitable for this purpose.

980. Hardening by Reagents. If large pieces of nervous tissue are to be hardened, it is necessary to take special precautions in order to prevent them from being deformed by their

weight during the process. The spinal cord or small portions of any region of the encephalon may be cut into thin slices, laid out on cotton or glass wool in a vessel into which the hardening fluid is poured. The specimens may also be suspended in the liquid. Another good plan consists in adding to the hardening fluid just enough glycerin or sodium chloride to make tissues float.

If several pieces are placed in the same vessel, they should never be put on top of each other. Voluminous organs to be hardened *in toto* should be at least incised as deeply as possible in the less important regions. With the exception of the dura mater, the membranes are not generally removed at first, as they serve to protect the tissues. They can be removed partially or entirely later on when the hardening has made some progress. In the case of material intended for Golgi's methods it is best not to remove them at all.

The *spinal cord, medulla oblongata* and *pons Varolii* may be hardened *in toto*, and the preparation hung up in a cylindrical vessel with a weight attached to its lower end to prevent it from becoming distorted. The dura should be slit up along the front and back of the cord and each half cut across in two or three places to prevent longitudinal shrinkage.

A useful method of treating a brain which is to be studied from an anatomical standpoint is to make sagittal incisions through the corpus callosum and infundibular recess, allowing the cerebrospinal fluid to drain away, and then to suspend the brain in 4 or 5 litres of formol saline by a string, either passed between the basilar artery and the pons, or passed through the cerebral hemispheres from side to side.

The action of most hardening fluids is greatly enhanced by heat. But in the judgment of most histologists this rapid hardening is not, as a rule, attended by good results, and one should have recourse to it only for particular reasons and special purposes after a tentative experiment at establishing the degree of temperature at which the desired results may be obtained without injuring the delicate structure of the nerve-tissue.

On the other hand, the hardening action at room temperature of certain reagents, such as solutions of chromic salts, proceeds so slowly that decomposition may set in before the fluid has had time to act effectively. For this reason voluminous preparations which are to be hardened *in toto* in solutions of chromic salts, and were not injected as described in § 978, should be put away in a very cool place or in an ice-chest. A human cerebral hemisphere may require eight or nine months for hardening in this way.

The volume of the fluid should always be very large in proportion to that of the pieces of tissue and to their number.

MARIE's method of fixing and hardening *in situ* is highly recommended; for its indications and contra-indications, see SAINTON and KATTWINKEL (*Deutsche Arch. klin. Med.*, lx, 1898, p. 548) and PFISTER (*Neurol. Centrbl.*, xvii, 1898, p. 643).

981. The Reagents to be Employed. As in the case of the fixation by injection one should bear in mind that the preservation of tissues for neuro-histological investigations greatly depends upon the purpose in view. Fixing and hardening fluids which are excellent for cytological investigations are very often unsuitable for anatomical methods. (See § 977.) On the other hand, material collected and prepared for cyto-architectural or fibro-architectural studies can hardly be used to elucidate questions regarding the intimate structure of nerve-cells or nerve-fibres. Alcohol, formalin * and chromic salts are most frequently used because they are generally ready at hand, and because they are useful for carrying out afterwards either a great number of methods or certain methods, under constant conditions of hardening and staining.

982. Alcohol. It is generally employed in the strength of 94 to 96 per cent., penetrates well and hardens quickly; but as it rapidly absorbs water from the tissues the latter shrink considerably, while the alcohol loses its fixing and hardening properties through hydration. It has consequently to be changed soon and used in quantities exceptionally large in proportion to the size of the pieces, which ought to be neither too small nor too large. For this reason one seldom hardens in alcohol voluminous organs, and its use has become on the whole very restricted. Alcohol, however, remains the principal fixing and hardening reagent for cytological investigations by Nissl's method (see § 1000), and for carrying out some of Ramón y Cajal's reduced silver processes, its shrinking influence being counteracted by having recourse for the first fixation to weaker dilutions of alcohol (60 to 70 per cent.) to be raised gradually up to 95 or 96 per cent. within the first nine to twelve hours, and to be changed once or twice or more often in the next few days.

983. Formalin. Since the time when it was introduced into histological technique by F. BLUM (*Ztschr. wiss. Mikr.*, x, 1893, p. 314); J. BLUM (*Zool. Anz.*, xvi, 1893, p. 450); HERMANN (*Anat. Anz.*, ix, 1893–94, p. 112); HOYER, jun. (*Anat. Anz.*, *Verf. Anat. Ses.*, ix, 1894, p. 236); LACHI (*Monit. Zool. Ital.*, v, 1895, p. 15) and many others, its use has been steadily increasing

* As is well known, commercial formalin is a 40 per cent. solution of formaldehyde; when in this and the following chapters on the nervous system a 5, 10 or 20 per cent. solution of formalin is mentioned, it is intended to mean 5, 10 or 20 parts of commercial formol, and 95, 90 or 80 parts of water, respectively, while, *e.g.*, a 20 per cent. solution of formaldehyde is the commercial formalin diluted with half its volume of water.

because of the many advantages it offers. As a matter of fact it penetrates more quickly than solutions of chromic salts, and even than alcohol; it is not likely to over-harden and it allows of the most various after-treatments and methods of staining.

Several writers have insisted that for nervous tissue it should not be acid, but some prefer it acid. See "Retina." For neurofibrils it should be preferably neutral. To neutralise it, it is generally sufficient to prepare its solutions with tap water, but one may shake it with some calcium or magnesium carbonate. Some authors prefer to neutralise it with ammonia. (See also § 113.)

The strength of the formalin solutions generally used for fixing and hardening nerve-tissues varies considerably with the quality of the material in hand, but particularly with the age of the subjects. As a rule the more delicate the material and the younger the subject, the weaker should be the formalin solutions to be employed at first. Generally one starts with a 3 or 5 per cent. solution in the case of very soft tissues, gradually increasing the strength up to 10 or 12 per cent. An adult human encephalon can be very well preserved in a 10 or 15 or 20 per cent. solution with two changes of the fluid during the first days of fixation and hardening. See further on this subject § 113.

For anatomical work and for almost all histological processes, 10 parts of commercial formalin and 90 parts of 1 per cent. sodium chloride form an excellent initial fixative. In spite of Nissl's own opinion, good staining of Nissl bodies can be obtained in human material after a few weeks' fixation by formol saline, especially if the formalin solution is kept neutral. In older material, washing the tissues in weak ammonia water before imbedding greatly improves the staining.

Stevenson has elaborated a method by which the bulk and weight of the brain is kept constant (*Arch. Neur. and Psych.*, vi, 1923, p. 763). It is found, however, that there is very little change in the brain weight after formol saline fixation.

Formalin can be associated with, or followed by, alcohol (§ 113) or other reagents. See Fish (*Trans. Am. Micr. Soc.*, xvii, 1895, p. 319).

Joré's fluid (see 8th edition) forms an excellent initial fixative.

Parker and Floyd (*Anat. Anz.*, xi, 1896, p. 156) advise for sheep's brains a mixture of 6 volumes of 95 per cent. alcohol and 4 volumes of 2 per cent. formol. Brains may be kept in the mixture for months.

Gerota (*Int. Monatschr. Anat.*, xiii, 1896, p. 124) treats fœtal brains by first injecting the vascular system with a 10 to 15 per cent. solution of formol in 85 per cent. alcohol, and then bringing the heads into 5 to 10 per cent. formalin; after one or two days

he removes the brains from the skull and puts them back for fifteen to twenty days into the formol.

KADYI (*Neurol. Centrbl.*, xx, 1901, p. 687) takes 5 parts of formol, 100 of water, and 2 of bicarbonate of soda, for four to ten days.

HERDLICKA (*Proc. U. S. Nat. Mus.*, xxx, 1906, p. 304) takes 3 parts of formol, 25 to 45 of water, and 72 to 52 of 95 per cent. alcohol.

LANDAU (*Ztschr. wiss. Mikr.*, xl, 1923, p. 22) recommends fixing large pieces, or even whole brains, in a mixture of 90 parts of 1 per cent. picric acid and 10 of formalin. The picric acid is removed afterwards by adding some lithium iodide to the alcohols of ascending strength used for dehydration.

The methods for removing the deposits which sometimes become formed in tissues preserved in formalin are described in § 113.

984. Chromic Salts. The most commonly used is potassium bichromate. The liquid of Erlicki has a more rapid action than other solutions of chromic salts, but it has been generally abandoned because of the alterations it very often produces.

SAHLI (*Ztschr. wiss. Mikr.*, ii, 1885, p. 1), after investigating the action of the usual solutions, concludes that the best hardening agent for fresh tissues is pure potassium bichromate, in 3 or 4 per cent. solution, the hardening being done in a cold place. He rejects the liquid of Erlicki on account of the precipitates to which it so frequently gives rise.

OBERSTEINER is of the same opinion, and recommends pure bichromate for general hardening purposes; whilst for the study of delicate structural details he recommends fixing in Fol's modification of Flemming's fluid (§ 47) for twenty-four hours, followed by washing with water and hardening in 80 per cent. alcohol.

In view of the slowness of penetration of chromic salts it is often advisable first to place the material for some days in 10 per cent. formalin in saline, and then transfer it into a 3 per cent. solution of potassium bichromate or Müller's fluid. But the formalin should be properly washed away and the bichromate solution changed after twenty-four hours, and again as soon as it becomes even slightly turbid.

Potassium bichromate should be employed at first in 2 or 2·5 per cent. solutions, to be replaced by more concentrated solutions up to 5 per cent. Da Fano recommends leaving jars in an incubator at 22° to 24° C. until the hardening is completed. Instead of pure potassium bichromate in solutions of increasing strength, Müller's fluid can equally well be employed throughout the process of hardening; it only needs to be changed from time to time.

Ammonium bichromate should at first be employed at half the strength recommended for potassium bichromate; it can subsequently be raised to as much as 5 per cent. for the cerebellum towards the end of the hardening.

See also BONVICINI (*Ztschr. wiss. Mikr.*, xxvi, 1909, p. 412).

RAWITZ (*ibid.*, p. 338) puts formol material for exactly five days into alcohol with 10 per cent. tinctura iodi P. G., then for eight to ten days into saturated solutions of potassium bichromate changed after the first day, and lastly into 95 per cent. alcohol for three days in the dark.

The methods of NISSL (*Enzykl. Mik. Techn.*, ii, 1910, p. 245), BETZ (*Arch. mikr. Anat.*, ix, 1873, p. 101), LEWIS (*Human Brain*), HAMILTON (*Journ. Anat. and Physiol.*, xii, 1878, p. 254), DUVAL (*Journ. de l'Anat.*, xii, 1876, p. 497), DEECKE (*Journ. R. Micr. Soc.*, 1883, p. 449), are no longer used.

985. Other Reagents. Small pieces can be fixed in *Zenker's fluids* or in *Zenker-formalin* (HELLY, MAXIMOW) as described in § 78. The pieces are best imbedded in paraffin. The sections can be stained by Mallory's eosin-methylen blue method (§ 342), Mann's methyl blue and eosin method (§ 356), Mallory's phosphotungstic acid-hæmatoxylin method for neuroglia and other methods.

Bouin's fluid (§ 115) can also be usefully employed for relatively small pieces, which can be imbedded either in celloidin or paraffin, the picric acid being extracted during dehydration by means of potassium or lithium iodide added to the weaker alcohols. Or after removal of the picric acid they are stained in bulk with hæmatoxylin and eosin as advised by DA FANO (*vide* § 999 and *Journ. Physiol.*, lx, 1925, p. 1) chiefly for spinal and sympathetic ganglia and the central nervous system of small animals. Especially beautiful differential staining with van Gieson's mixture can be obtained either after primary fixation or after-hardening in Bouin's fluid. Paraffin sections of the nervous system, fixed in this way, may be stained for neuroglia by Victoria Blue (Anderson's modification, *q.v.*). It is unnecessary to remove the picric acid from the block completely before cutting it. It may be removed from the section by treatment with warm distilled water or by a prolonged wash in alcohol.

Osmic acid can be employed alone only for very small pieces, but it is useful for special purposes; its penetrating power can be enhanced by keeping the vessels at temperatures varying between 20° and 40° C. (see further on this subject, § 40).

Chromic acid is rarely used alone. Its action is rapid, but uneven, and causes shrinkage and brittleness. A very little (*e.g.* 3 to 5 drops of a 1 per cent. solution to every 100 c.c. of fluid), added to bichromate solutions will do no harm and quicken the hardening.

Nitric acid has been and still is employed in strengths of 10 to 12 per cent.

Ten per cent. *neutral acetate of lead* affords, according to KOTLAREWSKI (*Ztschr. wiss. Mikr.*, iv, 1887, p. 387), an excellent preservation of ganglion cells.

Corrosive sublimate solutions either alone or mixed with other reagents (see Chapter V), have been very often used for cytological studies.

Similarly *acetic alcohol.*

MANN (*Ztschr. wiss. Mikr.*, xi, 1894, p. 480) recommends for cell studies the following fluids :—

(1) Heidenhain's saturated solution of corrosive sublimate in physiological salt solution ; (2) saturated watery solution of corrosive sublimate with 1 drop of nitric acid to every cubic centimetre of the sublimate solution ; (3) Heidenhain's sublimate solution with 1 grm. of picric acid and 1 of tannin ; (4) equal parts of 1 per cent. osmic acid and Heidenhain's sublimate solution.

MANN (*op. cit.*) for cell studies, puts small pieces for twenty-four hours into a solution of 5 parts of potassium iodide and 25 of iodine in 100 parts of water, and then into 70 per cent. alcohol.

OLMACHER (§ 70) recommends his mixture. KODIS (*Arch. mikr. Anat.*, lix, 1902, p. 212) fixes tissues in a saturated solution of cyanide of mercury, brings them into 10 per cent. formol, and makes sections by the freezing method.

NELIS (*Bull. Ac. R. Belg. cl. d. sc.*, xxxvii, 1899, p. 102) fixes ganglia for twenty-four hours in Gilson's fluid.

KING (*Anat. Rec.*, iv, 1910, p. 213), after trying over twenty-five methods on brains of rats, concludes that the best is Ohlmacher's. The brain should be put into it for two to three hours, then for one into 85 per cent. alcohol, then into 70 per cent. with iodine for at least twenty-four hours, then passed through alcohols of ascending strength and alcohol-ether into 2 per cent. celloidin for two to three days, and through chloroform and benzol into paraffin. In her opinion, Bouin's is the best of the formol liquids ; Tellyesniczky's is the only one of the bichromate mixtures that equals it. All sublimate mixtures fix the nuclei well, but vacuolise the cytoplasm.

986. MARINA'S Method (*Neurol. Centralb.*, xvi, 1897, p. 166) has the great advantage of allowing the subsequent carrying out of a great variety of staining methods. The material should be fixed in a mixture of 100 c.c. of 90 per cent. alcohol, 5 c.c. of formalin, and 0·10 grm. of chromic acid. On the following day the pieces are reduced in size and placed in some freshly-prepared fixing fluid for another two to five days. They are then gummed to wooden cubes, and these are preserved in 90 per cent. alcohol, to which about 1 per cent. of chromic acid may be added. The sections can be stained by Nissl's soap-methylen blue method § 1001) or with thionin, or by a modification of Held's method for neurosomes (§ 1005). If they are transferred into chromogen. they can be used for staining neuroglia fibres by Weigert's method (§ 1083) ; if mordanted as suggested, by Vassale (§ 1056), or in 3 per cent. potassium bichromate to which a few drops of ammonia have been added, they can be employed for staining medullated nerve fibres.

See further particulars on this subject in the original papers of TRZEBINSKI, *Virchow's Arch.*, cvii, 1887, p. 1 ; DIOMIDOFF, *ibid.*, p. 499 ; FISH, *The Wilder Quarter-Century Book*, 1893, p. 335 ; DONALDSON, *Journ. Morphol.*, ix, 1894, p. 123 ; TIMOFEEW, *Intern. Monatschr. Anat.*, xv, 1929, p. 259.

987. Nervous System of Reptiles, Amphibia and Fishes. MASON (*Central Nervous System of Certain Reptiles, etc.* ; WHITMAN'S *Methods*, p. 196) recommends iodised alcohol, six to twelve hours ; then 3 per

cent. bichromate, changed once a fortnight until the hardening is sufficient (six to ten weeks).

Burckhardt (*Das Centralnervensystem von Protopterus*, Berlin, 1892; *Ztschr. wiss. Mikr.*, ix, 1892, p. 347) recommends a liquid composed of 300 parts of 1 per cent. chromic acid, 10 parts of 2 per cent. osmic acid, and 10 parts of concentrated nitric acid, in which brains of Protopterus are hardened in twenty-four to forty-eight hours.

Fish (*Journ. of Morphol.*, x, 1895, p. 234) employed for *Desmognathus* a mixture of 1000 c.c. of 50 per cent. alcohol, 5 c.c. of glacial acetic acid, 5 grm. of corrosive sublimate, and 1 grm. of picric acid, fixing for twelve to twenty-four hours, and passing through the usual alcohols.

Strong (*Journ. comp. Neurol.*, xiii, 1903, p. 296) fixes (and decalcifies at the same time) the heads of young *Acanthias* in a mixture of 9 parts of 5 per cent. iron alum and 1 part of formol, for about two weeks, makes paraffin sections, stains with hæmatoxylin, and differentiates in 1 or 2 per cent. iron alum.

Johnston (*Gegenbaur's Morphol. Jahrb.*, xxxiv, 1905, p. 150) recommends for *Petromyzon* fixing in Zenker's fluid and imbedding in paraffin. The sections are stuck to slides and stained for several hours with alum carmine. After rinsing in water they are placed in a mixture arrived at by trial of saturated watery solution of nigrosin and saturated watery solution of picric acid plus 1 per cent. acid fuchsin.

IMBEDDING AND SECTION CUTTING

988. Imbedding is by no means always necessary, and is objected to in some cases. Indeed, sections can be made from any part of the central nervous system without it, if the tissues are well hardened. Material hardened in alcohol, or in chromic solutions, or treated according to Golgi's methods, may be glued on to a piece of wood or hard cork (or still better to a glass cube) by means of a rather thick solution of gum arabic or of celloidin. As soon as it begins to stick to the support the whole is put into 70 or 80 per cent. alcohol to harden the gum or the celloidin, and then fixed in the object-holder of the microtome and cut. Or one can simply make a clean cut at the bottom of the specimen, dry it with blotting paper and stick it on the support with sealing-wax or paraffin of high melting point. For section cutting the knife should be wetted with alcohol or water; if the latter is used, some soap may be added to it to prevent it from running into drops on the knife.

Formalin material is often cut by freezing methods, these being very largely used since the introduction of CO_2 microtomes, by means of which many and relatively very thin sections can be rapidly obtained with great economy of time and without imbedding media. Imbedding in gelatin may be resorted to in special cases. The blocks must then be cut by means of the freezing microtome and the sections mounted in glycerin jelly, Apáthy's syrup or some similar medium (§§ 463 *et seq.*).

Imbedding in paraffin is not advised for the nervous system in general, particularly after fixation in alcohol, and bichro-

mate solution. One should have recourse to it only for special cytological methods, taking care not to use paraffin of too high a melting point. Shrinkage is reduced to a minimum if the tissues are treated with Zenker's fluid for a few hours before being dehydrated.

Imbedding in celloidin is very largely used, and to great advantage for many purposes.

If, notwithstanding every precaution, the celloidin has not thoroughly penetrated the tissues, good sections may still be obtained by DUVAL's method of collodionising the sections. The cut surface of the block is dried by blowing on it, and is covered with a thin layer of collodion laid on it with a brush. As soon as this layer has somewhat dried, which happens very rapidly, a section is cut, and the cut surface collodionised as before, and so on for each section.

The above applies to section cutting of small, medium-sized and even relatively large pieces. Also unusually large pieces, entire human hemispheres, and brains of high vertebrates can be cut into relatively thin and, if necessary, serial sections after imbedding either in celloidin or paraffin or by mixed methods. The processes used for the purpose do not differ essentially from those above-mentioned and fully described in Chapters IX and X, but (particularly for cyto-architectural and fibre-architectural studies) special apparatus and installations are needed, the description of which is outside the province of this book.

See also CIAGLINSKI, *Ztschr. wiss. Mikr.*, viii, 1891, p. 19 ; FEIST, *Ztschr. wiss. Mikr.*, viii, 1891, p. 492 ; DEFECKE, *op. cit.* ; DEJERINE, *Anat. Centres Nerveux*, i, 1895, pp. 1–57 ; STRASSER, *Ztschr. wiss. Mikr.*, ix, 1892, p. 1 ; BRODMANN, *Journ. Psychol. u. Neurol.*, ii, 1903–4, p. 206 ; WARNKE, *ibid.*, p. 221 ; LIESEGANG, *Ztschr. wiss. Mikr.*, xxvii, 1910, p. 369 ; VENDEROVIC, *Anat. Anz.*, xxxix, 1911, p. 414.

989. Numbering in Series Sections from Celloidin Blocks. Besides the methods described in §§ 216 *et seq.*, the following one by DA FANO (*Proc. Physiol. Soc.*, *Journ. Physiol.*, lx, 1925, p. 13) has been found extremely useful whenever the sections can be mounted without dissolving the celloidin. Pieces of any desirable size are imbedded in celloidin by means of paper boxes or any similar device which may allow one to arrange them according to the way in which the series will have, afterwards, to progress. At the same time care should be taken that the pieces are surrounded by an amount of celloidin sufficient for subsequently writing a progressive number on the sections. The celloidin blocks are stuck to appropriate supports, and these are fixed on the microtome as usual. As each section is cut it is spread flat on the knife and the free celloidin at one corner dried with blotting paper. Without any loss of time a number is written

on this corner by means of a small good brush and a mixture of 10 c.c. of Indian ink and 3 c.c. of equal parts of anhydrous ether and acetone. The figures dry up instantaneously and become almost engraved in the celloidin. The sections are then transferred to a dish of 60 to 70 per cent. alcohol. The numbers thus written on the celloidin are not deleted by water and common reagents, such as alcohols, up to 96 per cent., xylol, the Weigert mordant, the bath used for toning and fixing Golgi-Cox specimens and so on. Many sections can, therefore, be stained and treated as desired at the same time and finally mounted according to the progression of their numbers, provided, of course, that mounting media which dissolve celloidin are not used.

GENERAL STAINS

990. Carmines. Ammonia-carmine is good for general views. Stain very slowly in dilute solutions. Bichromate material should be brought direct into the stain without passing through alcohol (see § 56).

Picro-carmine has much the same action, but gives a better demonstration of non-nervous elements.

BOLLES LEE (1913 Ed.) preferred carmalum with formol material as giving a more delicate stain. He found it better than para-carmine.

The best way of staining formol material with ammonia-carmine, carmalum, picro-carmine and the like, consists in making frozen sections, transferring them for a few hours either to Müller's fluid, or 0·5 per cent. chromic acid as suggested by SCHWALBE (*Centralb. allg. Pathol.*, xii, 1901, p. 881). Sections are then washed for a longer or shorter time according to the amount of mordant one wishes to extract, proceeding afterwards to stain with one of the above-mentioned carmine solutions.

On the other hand, sections of non-imbedded material fixed and hardened in one or other of the fluids mentioned in §§ 982, 484 may be stained not only with carmines, but also with a great variety of dyes if one so desires (see Chapter XVII:). The same applies to sections of imbedded material, though the after-treatment to which it has been submitted may render more or less difficult the carrying out of certain general stains. One should remember that in any case the results thus obtained are not very instructive, and by no means comparable with those attainable by the rational use of the special methods described in the following chapters.

991. Anderson's Alum Carmine (*Journ. Path. and Bact.*, xxix, 1926, p. 117). The following carmine solution has recently been proposed for counterstaining Weigert-Pal preparations (§ 1058), but can be used also for general purposes. Put 1 grm. of pure carmine in a 200 c.c. flask and add 10 c.c of absolute alcohol :

mix thoroughly. Add 5 c.c. of a 2 per cent. solution of chloride of lime and mix again. Add 90 c.c. of a saturated solution of ammonia alum and shake well. Bring the mixture to the boiling point, shaking several times while it is heating; boil for one minute and filter. After filtering and cooling add 5 c.c. of acetic acid and keep the stain in a well-stoppered bottle. It seems to last indefinitely; it should be filtered back into the bottle after use. Sections, which were stained and differentiated by the Weigert-Pal method, are, according to Anderson, washed in water and placed in the carmine stain for two to three hours at 50° C. They are then washed until the celloidin is a faint pink colour, dehydrated and mounted in the usual way. As the carmine solution acts like a weak differentiator of the hæmatoxylin, it is as well not to carry the decolourisation by Pal's method quite so far as usual. This carmine preparation can be employed with advantage for a variety of purposes. Except in the case of material treated with bichromate, there is no necessity for keeping the sections so long at 50° C.; warming for thirty minutes at 37° C. is in most cases sufficient.

For other carmine processes of staining, see SCHMAUS (*Münch. med. Wochenschr.*, xxxviii, 1891, p. 147); UPSON (*Neurol. Centralb.*, vii, 1888, p. 319); FREEBORN (*Journ. Roy. Mic. Soc.*, 1889, p. 305); KADYI (*Neurol. Centralb.*, xx, 1901, p. 687); CHILESOTTI (*Centralb. allg. Pathol.*, xiii, 1892, p. 193).

992. **Nigrosin and Anilin-blue-black.** Nigrosin has given good results in some hands. Anilin-blue-black has been much recommended by SANKEY (*Lancet*, ii, 1875, p. 82). LEWIS (*Human Brain*, London, 1880, p. 125, and *Quart. Journ. Micr. Sc.*, xvi, 1876, pp. 73–75); VEJAS (*Arch. f. Psych.*, xvi, 1885, p. 200); JELGERSMA (*Ztschr. wiss. Mikr.*, iii, 1886, p. 39); SCHMAUS (*Münch. med. Wochenschr.*, xxxviii, 1891, p. 147), and others. And see also previous editions.

993. **Picronigrosin.** MARTINOTTI (*Ztsch. wiss. Mikr.*, ii, 1885, p. 478) stains for two or three hours or days in a saturated solution of nigrosin in saturated solution of picric acid in alcohol, and washes out in a mixture of 1 part of formic acid with 2 parts of alcohol.

994. KAISER (*Ztschr. wiss. Mikr.*, vi, 1889, p. 471) stains sections of spinal cord for a few hours in a solution of 1 part of *naphthylamin brown*, 200 of water, and 100 of alcohol, washes with alcohol, clears with origanum oil and mounts.

995. **Alizarine.** SCHRÖTTER (*Neurol. Centralb.*, xxi, 1902, p. 338) stains sections for twenty-four hours in a 1 to 2 per cent. solution of sulphalizarinate of soda, differentiates for half to one minute in tap-water, dehydrates, and mounts. This is a general stain, but demonstrates Nissl's bodies and other details.

996. MALLORY'S **Phosphomolybdic Acid Hæmatoxylin and** KODIS' *modification*, see § 311.

997. **Hæmatoxylin and Acid Fuchsin.** FINOTTI (*Virchow's Arch.*, cxliii, 1896, p. 167) stains in hæmatoxylin, counterstains for three minutes with 0·5 to 1 per cent. solution of acid fuchsin, and differentiates in 75 per cent. alcohol containing a very little caustic potash.

VAN GIESON'S hæmatoxylin and picro-fuchsin (§ 326) gives useful general views of nervous tissue.

Its special value for the nervous system lies in the fact that it differentiates neuroglia from collagen fibres and so outlines sharply the limits of the blood-vessels and meninges. For the use of this stain on celloidin sections we recommend the following technique: Sections of 8 to 10μ are transferred from spirit to water and stained for two to five minutes in Weigert's iron hæmatoxylin, or in Hansen's iron-alum hæmatoxylin. They are then thoroughly washed in alkaline tap-water and differentiated in acid alcohol if necessary. (Up to this point several sections may be treated at a time but from this stage on they must be treated individually.) Sections are passed into van Gieson's counterstain by means of a fine bent glass-rod, and moved about in the stain for eight to twelve seconds (here careful timing is necessary), then washed quickly in distilled water or in tap-water made acid with acetic acid, washed again quickly in 70 per cent. alcohol, and transferred to 95 per cent. alcohol, where they are left for from five to fifteen minutes. This wash in alcohol removes the picric acid from the nuclei, leaving them clear blue. They are then cleared in carbolxylol, washed in xylol and mounted in xylol balsam saturated with salicylic acid crystals (Roussy & Lhermitte).

It is important that after van Gieson's counterstain the sections should not enter any alkaline solution. If mounted in acid balsam, the red staining of the fuchsin is permanent and the salicylic acid does not appear to cause the hæmatoxylin to fade.

Rawitz (*Ztschr. wiss. Mikr.*, xxvi, 1909, p. 341) has some complicated methods with *Indulin*, *Indaminblau*, and *Azosaureblau*, which take twenty-eight days; and (*ibid.*, xxviii, 1911, p. 1) others with fuchsin and azofuchsin which take over thirty-six days.

Ariëns Kappers (*ibid.*, xxviii, 1911, p. 417) describes a staining method with extract of elderberries for material fixed and hardened in Müller's fluid or similar solutions. It is very simple and particularly recommended for photographic purposes; it should be carried out as follows: Stain celloidin or paraffin sections overnight in neutralised elderberries extract (obtained by fermentation at 20° to 25° C.), to which 1 per cent. carbolic acid has been added. Wash in water. Differentiate in 3 per cent. *Liquor ferri sesquichlorati* P. G., wash, dehydrate, and mount.

998. Methods for Staining in Bulk. Pick (*Centralb. f. Gynakol.*, xxii, 1898, p. 227) suggested staining and fixing frozen sections at the same time in a mixture of 10 per cent. formalin and alum carmine.

Schridde and Fricke (*Centralb. f. allg. path. u. path. Anat.*, xvii, 1906, p. 720) fix small pieces of tissue in 9 parts alum carmine with 1 part formalin for three to four days or longer, wash for twelve to twenty-four hours and differentiate in 200 parts of 75 per cent. alcohol with 1 part of 25 per cent. ammonia.

Rothig (*Folia Neurobiol.*, ii, 1909, p. 385) fixes and stains for about four weeks in saturated solution of methylenazur I.

in 10 per cent. formol, put for ten to fifteen minutes into acetone, then for twelve hours into chloroform, and imbeds in paraffin. He has also a process with trichloracetate of lead and methylenazur.

Strecker (*Zeitschr. wiss. Mikr.*, xxviii, 1911, p. 17) recommends equal parts of 10 to 20 per cent. formalin and of Erhlich-Biondi tri-acid stain for twenty-four to forty-eight hours. And also 3 parts of toluidin blue in 100 parts of 10 per cent. formalin, fixing the blue thereafter with Bethe's ammonium molybdate solution.

Ilberg (*Neur. Centralb.*, xv, 1896, p. 831) fixes in 96 per cent. alcohol, and stains pieces in Nissl's soap solution of methylen blue for five to ten days, differentiating in 96 per cent. alcohol for two to three days, changing it every day. Imbed in paraffin and further differentiate the sections, if necessary, in 96 per cent. alcohol.

999. Da Fano (*Journ. Physiol.*, lx, 1925, p. 1) fixes small pieces of fresh tissue in Bouin's fluid, transfers to 70 to 80 per cent. alcohol overnight and then places in fresh alcohol of the same strength to which is added 1 per cent. lithium iodide, renewing this every day till the picric acid is almost entirely removed. Wash overnight in running water and then for some hours in distilled water, and transfer to Scott's modification of Ehrlich's hæmatoxylin diluted with 3 parts either of distilled water or of 2 per cent. acetic acid. (Scott's formula (*J. Path. & Bact.*, xvi, 1911–12, p. 390), glycerol 100 c.c., water 100 c.c., alcohol (96 per cent.) 100 c.c., glacial acetic acid 10 c.c., potash alum 7 grm., hæmatoxylin 1 to 1·25 grm.; ripen several months.) Leave in this for two to three weeks according to thickness of pieces, changing the hæmatoxylin at the end of the first week. Wash quickly and put for two to three hours into acid alcohol (1 per cent. HCl in 70 per cent. alcohol). Wash overnight in tap-water. Pass through the alcohols up to 95 per cent. and thence place in 1 per cent. eosin in 95 per cent. alcohol for one week. Wash in 95 per cent. alcohol for two to twenty-four hours. Dehydrate, clear and imbed in paraffin.

Cummings (*J. Comp. Neur.*, xxxviii, 1925, p. 401) fixes and stains the membranous labyrinth of the rat as follows: Fix for twenty-four to forty-eight hours in a modified Held's fluid. (Formalin 4 parts, glacial acetic acid 5 parts, 3½ per cent. pot. bichromate 91 parts.) Wash for twenty-four hours in running water, dehydrate up to 80 per cent. alcohol and decalcify in 3 per cent. HNO_3 in 80 per cent. alcohol. Pass through gradually weaker alcohols to 25 per cent. alcohol and then stain in diluted Delafield's hæmatoxylin or Mann's methyl blue eosin for three to five days. Dehydrate, adding a drop of ammonia to the alcohol, infiltrate with celloidin and cut by dry cedar oil method.

CHAPTER XL

NERVOUS SYSTEM—SPECIAL METHODS CHIEFLY CYTOLOGICAL *

A. METHODS FOR CELLS, DEMONSTRATING TIGROID SUBSTANCE AND OTHER GRANULAR MATERIALS

1000. Tigroid substance or bodies, chromophilic or chromatophilic substance or material or granules, Nissl's bodies or granules, etc., are all denominations for a markedly basophil substance which appears as blocks, granules or irregular patches within the cytoplasm of nerve-cells under certain conditions of fixing and staining.

It is now universally admitted that this substance exists in the living cells as a fluid or semi-fluid "plasm rich in nutritive value," and that the blocks, granules or patches are appearances chiefly due to the coagulation of this plasm, as brought about by the fixing agents employed for their demonstration. As, however, these bodies or granules appear always the same under constant optical conditions in healthy cells fixed and stained in a constant manner, they are said to be the equivalent of such healthy cells during life. "It follows that if the cells, prepared by the same method and examined under the same conditions, show a difference from the equivalent or symbol of healthy cells, the difference is the measure of some change that has occurred during life." (Halliburton, "Handbook of Physiology," London, 1920, p. 194. See also Einarson (*Am. Journ. Path.*, viii, 1932, 295).)

This is pointed out to make it quite clear why Nissl has always insisted that his method should be carried out according to his suggestions, and in a constant manner. At first (*Neurol. Centralb.*, iv, 1885, p. 500) he used to stain sections of material fixed in alcohol with a warmed watery solution of magenta or dahlia violet or vesuvine, and to differentiate them with alcohol. Later (*Allg. Ztschr. Psych.*, xlviii, 1892, p. 197) he suggested floating sections on a warmed solution of methylen blue (B patent), with subsequent differentiation with a 10 per cent. solution of anilin oil in 96 per cent. alcohol. The present form of the method was published in 1894 (*Neurol. Centralb.*, xiii, p. 507); but Nissl continued to introduce into it slight modifi-

* Revised by J. G. G. and R. O. S.

cations, to which due attention was paid when preparing the following account.

The Nissl method is extremely useful for the study of nervous tissue under physiological and pathological conditions ; it stains, when properly carried out, not only the tigroid substance and the basophil parts of the nuclei of nerve-cells, but also the nuclei and part of the cytoplasm of neuroglia cells as well as connective tissue elements normally or abnormally present in the nerve-tissue. In a somewhat modified form it has been largely and successfully employed for investigating the topographical distribution of nerve-cells (cyto-architecture) in different regions of the central nervous system of man and animals.

1001. Nissl's Methylen-blue Method. Not too small pieces of fresh tissue are fixed in 96 per cent. alcohol and hardened therein for a few days. They should not be allowed to fall to the bottom of the bottle, but kept floating by means of some filter paper or cotton-wool. The alcohol must be in large quantity in proportion to the number of pieces, and repeatedly changed. The pieces are cut without imbedding, and the sections collected in 96 per cent. alcohol, from which they are directly floated on some stain filtered into a watch-glass at the moment of using it. The stain should be at least three to four months old, and shaken at the moment of filtering the quantity needed. It is prepared by carefully dissolving 1·75 grm. of Venetian soap in 1 litre of distilled water and then adding 3·75 grm. of methylen blue (B patent). It is a good practice to shake the bottle vigorously from time to time, and to refilter into it the stain left in the watch-glass after staining one or more sections.

The watch-glass containing the stain with the section floating on it is warmed carefully over a flame until small bubbles rise to the surface. The section, which should not have fallen to the bottom of the watch-glass, is immediately transferred into a mixture of 10 parts of anilin oil and 90 parts of 96 per cent. alcohol, and as soon as no more colour is given off (if often takes only some seconds), it is lifted on to a slide, pressed with smooth filter paper, and cleared with a few drops of pure anhydrous cajeput oil. Care should be taken not to dry the section excessively with the filter paper and to pour the cajeput oil on to the section very quickly. The cajeput oil not only clears the section, but stops the differentiation ; it is, therefore, advisable to renew it after a little while on the section. As soon as this has become quite transparent, the cajeput oil is dried off with filter paper, the section thoroughly washed with benzol and covered with a drop of thick xylol-colophonium, rendered more fluid by passing the slide carefully over a flame, and quickly covering the section with a thin cover-glass before the colophonium sets again by cooling.

1002. Suggestions Regarding the Carrying-out of Nissl's Method. For the fixation of tissues, alcohol, formalin, sublimate or mixtures of these may be employed. The results are, however, better if tissues are placed for some time in alcohol after fixation with one of the above fluids. Acid solutions or chrome salts should be avoided. Old formalin material can be used for carrying out the Nissl method and its modifications if it is treated for twenty-four hours with ammoniated tap-water before being transferred to alcohols.

Although the bichromates of potassium and ammonium and all mixtures containing chromic salts should be avoided for cytological investigations in Nissl's sense, sections from material fixed for a short time (twenty hours) in the mixtures of Orth, Helly or Maximow are well stained by Giemsa's fluid if this is employed as described in § 877, and p. 400.

Sections of material which were not fixed in alcohol and of imbedded tissues, however fixed, stain, as a rule, very poorly by Nissl's soap-methylen-blue method; but excellent results can be obtained by staining such sections with watery solutions (generally 0·5 to 1 per cent.) of toluidin blue, thionin, Unna's polychrome methylen blue, cresyl violet, dahlia violet, vesuvine, neutral red, magenta red, Azur II, and the like.

Particularly good results are sometimes obtained by restaining the sections a second and a third time after having differentiated them completely each time (Da Fano, *Proc. Physiol. Soc.*, *Journ. Physiol.*, liv, 1920–21, p. 114).

All specimens stained by Nissl's method keep badly, but they can be preserved practically unaltered for months or even years if the following points are observed: (1) the anilin alcohol or alcohol used for differentiation purposes should be pure and completely removed by means of pure benzol before mounting the sections; instead of benzol, pure xylol can be used, though not advised by Nissl; (2) the xylol-colophonium should be rather thick and prepared with pure xylol; instead of colophonium, Canada balsam can be used; it is in any case important that either the colophonium or the balsam should not be acid; some authors prefer thick cedar-wood oil or similar mounting media; (3) the mounted specimens must be carefully protected from light.

1003. Modifications of Nissl's Method. Bielschowsky and Plien's **Cresyl Violet Method** (*Neurol. Centrbl.*, xix, 1900, p. 1141). Celloidin or paraffin sections of material fixed either in alcohol or formalin, or sections made by the freezing method from formalin material, are stained for twenty-four hours in a very diluted solution of cresyl violet R.R., prepared by adding 6 to 8 drops of a concentrated aqueous solution to every 50 c.c. of distilled water. After a quick wash in distilled water sections are brought

through the ascending series of alcohols, cajeput oil, and xylol into balsam. The preparations keep better than those stained with thionin or toluidin blue, and are particularly useful for photographic purposes and cyto-architectural studies. The cresyl violet R.R. can also be used in 0·5 to 1 per cent. watery solutions; paraffin sections stuck to slides are well stained after some hours, when they are differentiated in alcohol and mounted in the usual way.

REHM (*Munch. med. Wochenschr.*, xxxix, 1892, p. 217) floats sections for half a minute to a minute on a hot 0·1 per cent. solution of methylen blue, differentiates them in 96 per cent. alcohol, and clears them with origanum oil.

LUGARO (*Rev. Neurol. and Psych.*, iii, 1905, p. 339) fixes for forty-eight hours in 5 per cent. nitric acid in absolute alcohol, imbeds in paraffin, and stains sections for several hours stuck to slides in 1 : 2000 or 1 : 3000 toluidin blue; after a quick wash the stain is fixed by means of a 4 per cent. solution of ammonium molybdate; wash, dehydrate, clear in xylol, and mount in balsam.

LENHOSSÉK (*Fein. Bau d. Nervens.*, 1895) stains sections from formol material in a concentrated watery solution of thionin, rinses them with water, and mounts them with Nissl.

JULIUSBURGER (*Neurol. Centrbl.*, xvi, 1897, p. 259) stains sections of materials fixed in Orth's fluid and imbedded in celloidin, either with Nissl's methylen blue or with warmed neutral red.

ROSIN (*Deutsche med. Wochenschr.*, xxiv, 1898, p. 615) prefers neutral red for sections of material fixed in formalin.

LENHOSSÉK (*Neurol. Centrbl.*, xvii, 1898, p. 577) fixes ganglia either in Carnoy's fluid or in equal parts of a concentrated solution of corrosive sublimate and a saturated watery solution of picric acid. He imbeds either in paraffin or celloidin and stains the sections in a concentrated watery solution of toluidin blue overnight, rinses with water, differentiates quickly with alcohol, and clears with xylol or carbol xylol.

VAN GEHUCHTEN and NELIS (*La Cellule*, xiv, 1898, p. 374) recommend fixing spinal ganglia in Gilson's mixture, and staining thin paraffin sections in a watery solution of toluidin blue.

VAN GEHUCHTEN uses paraffin sections mounted on slides by the water method, and stains them for five to six hours in Nissl's methylen blue solution in the incubator at 35° or 40° C.

GOTHARD (*C. R. Soc. Biol.*, v, 1898, p. 330) stains celloidin sections for twenty-four hours in Unna's polychrome methylen blue, and differentiates them with a mixture of 5 parts of creosote, 4 of cajeput oil, 5 to 8 of xylol, and 16 of absolute alcohol. The mixture is removed with absolute alcohol and sections mounted in dammar after clearing with cajeput oil.

LUITHLEN and SORGO (*Neurol. Centrbl.*, xvii, 1898, p. 640) differentiate in Unna's glycerol-ether mixture after staining with polychrome methylen blue. They remove the differentiating mixture with absolute alcohol and clear with origanum oil.

Similarly LENNHOFF (*ibid.*, 1910, p. 20); or, polychrome methylen blue two minutes, distilled water quickly, carbol-pyronin-methyl green twenty minutes; distilled water quickly, absolute alcohol, oil, balsam.

LORD (*Journ. Ment. Sc.*, xliv., 1898, p. 693) makes sections from frozen fresh tissues, treats them for a few seconds with a mixture of equal parts of 6 per cent. formaldehyde and saturated solution of picric acid,

then rinses them with distilled water and stains them in 5 per cent. solution of methylen blue, B pat.

Luxenburg (*Neurol. Centrbl.*, xviii, 1899, p. 633) stains paraffin serial sections either with Nissl's soap methylen blue or with thionin, as Lenhossék.

Polumordwinow (*Ztschr. wiss. Mikr.*, xvi, 1899, p. 371) fixes in Gilson's mixture and uses for staining 1 part of a 1 per cent. solution of toluidin blue to 119 of distilled water alkalised with 1 of sodium carbonate.

Picromarine has been successfully used by Messner (*Journ. Psychol. Neurol.*, xviii, 1912, p. 204; xx, 1913, p. 256). Sections of alcohol material, imbedded in celloidin or not, are washed in water and then stained for five minutes in a warm diluted solution of Ranvier's picro-carmine. After a quick wash, they are differentiated in 3 per cent. hydrochloric acid, dehydrated, and mounted as usual. In the case of the spinal cord, medulla oblongata, and pons, the method also succeeds if material was fixed in formalin.

Einarson (*Am. Journ. Path.*, viii, 1932, p. 295), after experimenting with many dyes, recommends gallocyanin as the best stain for Nissl granules. A variety of fixatives may be used; 96 per cent. alcohol (five to six days), alcohol-formalin (twenty-four to forty-eight hours), sublimate-formol (twenty-four hours), formol-alcohol-sublimate (eight to twelve hours), Zenker with acetic acid (about twenty hours) and neutral formalin (20 per cent. for three to four days) all give good results. Sections are cut in paraffin and are stained in gallocyanin 0·3 grm., chromalum 10 grm., distilled water 200 c.c.

1004. Other Modifications and Methods for the Basophil Substance of Nerve-cells. See Farrar, *Rev. Neurol. and Psychiat.*, iii, 1905, p. 578; Ilberg, *Neurol. Centrbl.*, xv, 1896, p. 831; Goldscheider u. Flatau, *Norm. u. path. Anat. d. Nervens*, Berlin, 1898, or *Ztschr. wiss. Mikr.*, xvi, 1899, p. 102, and Nissl's remarks thereon, *Deutsche Ztschr. Nervenheilk*, xiii, 1899, p. 348; Cox, *Anat. Hefte*, x, 1898, p. 98; *Int. Monatschr. Anat.*, xv, 1898, p. 216; Auerbach, *Monatschr. Psychiat. u. Neurol.*, iv, 1898, p. 31; Myers, *Anat. Rec.*, ii, 1908, p. 434; Savini, E. u. Th., *Centrbl. Bakt.*, *I. Abth.*, xlviii, 1909, p. 697; Mosse, *Arch. mikr. Anat.*, lix, 1902, p. 403; Mentz v. Krogh, *Centrbl. Bakt.*, *I Abth.*, Orig., lviii, 1911, p. 95; Johnston, *Anat. Rec.*, xi, 1916, p. 297.

1005. Neurosomes. This term was first used by Held to indicate certain granules which since 1895 he succeeded in staining in nerve-cells by means of the method described hereafter. Two years later he included under the same heading minute, sharply-delineated rods of fairly uniform size which stained by the acid fuchsin method of Altmann and the iron-hæmatoxylin methods of Heidenhain and Benda. These rod-shaped bodies were afterwards identified by Cowdry (*Int. Monatschr. Anat.*, xxix, 1913, p. 473) as mitochondria, but the term "neurosomes" was retained to indicate the granules which can be shown by Held's erythrosin-methylen-blue method or one or other of its modifications. As pointed out by Cowdry, they are irregular in size and shape and more numerous in the cone of origin of the axon. They are seldom, if ever, seen after fixation in fluid containing osmic acid, which is most suitable for mitochondria, while they are best stained after fixing in Carnoy's fluid, picro-sulphuric acid and

other acid solutions which destroy mitochondria. Although it has been suggested by HEIDENHAIN (*Plasma und Zelle,* Fischer, Jena, 1911, p. 1110) that the neurosomes may be neurofibrils stained discontinuously, their precise nature is considered at present as unknown.

HELD'S **Methylen Blue and Erythrosin Method** (*Arch. Anat. Phys., Anat. Abth.*, 1895, p. 399; 1897, p. 226). Material may be fixed in alcohol, but preferably either in picro-sulphuric acid, or in van Gehuchten's mixture of alcohol, chloroform and acetic acid, or in 1 per cent. corrosive sublimate in 40 per cent. acetone. Tissues should be carefully imbedded in paraffin and the sections stuck to slides by the water method. They are stained with the aid of gentle heat for one to two minutes in a solution of 1 grm. erythrosin in 150 of distilled water acidulated with 2 drops of glacial acetic acid. After washing with water, the slides are transferred into a mixture of equal parts of Nissl's methylen-blue solution and 5 per cent. acetone, warming until all odour of the latter has disappeared. Differentiation is carried out after cooling by means of a 0·1 per cent. solution of alum until sections are reddish. Rinse in distilled water, dehydrate as rapidly as possible in absolute alcohol, wash in xylol and mount in balsam.

1006. Other Methods for the Double Staining of Nerve-cells. ROSIN (*Neurol. Centrbl.*, xii, 1893, p. 803) fixes the tissues by preference in alcohol or formalin, and imbeds either in paraffin or celloidin. Paraffin sections are stained for five minutes in a mixture of 0·4 grm. of Ehrlich's triacid (powder), 100 c.c. of distilled water, and 7 c.c. of a 0·5 per cent. solution of acid fuchsin. Celloidin sections are stained for one minute in a solution consisting of 4 parts of the above mixture and 1 part of 0·5 per cent. acid fuchsin. In both cases the sections are washed in distilled water, differentiated in 1 : 2000 acetic acid, dehydrated with absolute alcohol, cleared in xylol and mounted in balsam.

MANN (*Ztschr. wiss. Mikr.*, xi, 1894, p. 489) recommends the following for material fixed in one or other of the (watery) solutions mentioned in § 985. Paraffin sections stuck to slides by the albumen method are treated with Gram's solution of iodine in potassium iodide and washed in water. The still yellow sections are stained for five to ten minutes in 1 per cent. eosin in water, washed once more, and restained for twenty to thirty minutes in a 0·5 per cent. solution of toluidin blue in water. Rinse in water, dehydrate in absolute alcohol, clear in xylol, mount in turpentine-balsam. Instead of toluidin blue 4 per cent. of Ehrlich's methylen blue for intra-vitam staining in ¾ per cent. NaCl can be used.

B. METHODS FOR CELLS AND FIBRES DEMONSTRATING NEUROFIBRILS

1007. Introduction. Nerve-cells contain, in addition to the granular constituents already dealt with, a so-called achromatic portion apparently consisting of very fine fibrils which can be seen with great difficulty in the unstained state, but can be

brought into view by means of the methods described in the following paragraphs. These can be used with some slight variations, also for demonstrating neurofibrils in the axis cylinders of nerve-fibres as well as in nerve-endings.

1008. Apáthy's **Methods.** The *gold method* ("*Nachvergoldung*") has been given in § 410. The stain is very sharp, but good results are obtained only in certain invertebrates, and even in these with considerable difficulty.

The *hæmateine method* (*Mitth. Zool. Stat. Neapel*, xii, 1897, p. 712) has the same advantages and disadvantages, and has been little used since the discovery of the Cajal and Bielschowsky processes. Material may be fixed with corrosive sublimate, Zenker's fluid, picro-sulphuric acid, or any other mixture which is not inimical to staining with alum hæmatoxylin, and should be preserved in 90 per cent. alcohol. Pieces, no more than ½ cm. thick, are stained for at least forty-eight hours in hæmateine I.A. (§ 306), and then washed up to twenty-four hours in absolutely pure distilled water, or preferably suspended therein. Before the stain has become washed out of the neurofibrils entirely, it is fixed by putting the pieces for three to five hours into spring water, after which they are put back for not more than two hours into distilled water, dehydrated as rapidly as possible by hanging them up in absolute alcohol, and imbedded in paraffin or celloidin, after clearing with chloroform, and carefully protecting them from light whilst in chloroform or celloidin. The sections are mounted either in a resin or in neutral glycerin.

1009. Bethe's **Molybdenum-Toluidine Blue Method** (*Ztschr. wiss. Mikr.*, xvii, 1900, p. 13). Pieces of the central nervous system of vertebrates are fixed for twenty-four hours in 3 to 7·5 per cent. nitric acid, and then brought directly into 96 per cent. alcohol for a day or longer. They are afterwards put for twelve to twenty-four hours in a mixture of 1 part of ammonia (sp. gr. 0·95) with 3 of distilled water and 8 of 96 per cent. alcohol; for six to twelve hours into pure alcohol; for twenty-four hours into a mixture of 1 part of concentrated hydrochloric acid, 3 of distilled water, and 8 to 12 of alcohol; for ten to twelve hours into pure alcohol; for two to six hours into water. They are now mordanted with 4 per cent. ammonium molybdate, washed again, dehydrated and imbedded in paraffin. The sections, 8 to 10 μ thick, are seriated on slides by means of egg albumen, but *without water*, then passed through xylol and alcohol and differentiated, viz., covered with water poured on the sections so as to form over them a layer 1·5 to 2 mm. deep, and put into an incubator at 55° to 60° C. for ten minutes. They are then rinsed with water, covered with a 1 : 3000 solution of toluidine blue, stoved for another ten minutes, rinsed with water, and lastly treated with 96 per cent. alcohol till no more colour comes away. After dehydration with absolute alcohol they are mounted in the usual way.

The method is also applicable to invertebrates for which other fixing agents besides nitric acid are admissible, and the impregnation with ammonium molybdate may be carried out on the sections.

Lugaro's **Modification** (*Riv. Pat. Nerv. Ment.*, x, 1905, p. 265):

1. Fix in 1 per cent. nitric acid in pure acetone, forty-eight hours.

2. Acetone twelve to twenty-four hours, changing it three to four times.

3. Pass through equal parts of acetone and xylol into pure xylol till clear. Imbed in paraffin. Cut at 5 μ; fix to slides.

4. After dissolving out the paraffin with xylol, put the slides in absolute alcohol for twenty-four hours.

5. One per cent. acetic anhydride in absolute alcohol twenty-four hours.

6. Wash in distilled water and stain in 1 in 3000 toluidin blue for one hour at 55° to 60° C.

7. Wash in water and treat with 4 per cent. ammonium molybdate, as in Bethe's method.

8. Wash again, dehydrate in alcohol, clear in xylol, and mount in Canada balsam.

1010. Donaggio's **Methods** (*Riv. Sper. Freniatr.*, xxx, 1904, p. 397, and xxxii, 1906, p. 394). There are five methods of Donaggio. By the first two, pieces are stained in bulk before imbedding, but results are not so good as by the other three, the most important of which is Method III.

Method I. Fix in saturated solution of mercuric chloride; wash this out first with water to which a little tincture of iodine has been added, and then in distilled water for two to three hours. Place in pyridin for forty-eight hours, changing once, and, without washing, stain for forty-eight hours in 1 in 10,000 or 1 in 15,000 solution of thionin blue in distilled water. The pieces should be kept floating in the stain by being fixed to pieces of cork by one corner by means of paraffin wax. The stain should be changed once during this time. Pass directly into 4 per cent. ammonium molybdate solution containing 4 drops of pure HCl for each 100 c.c., and leave in this for twenty-four hours. Wash out in water for twelve hours, pass through alcohol and xylol to paraffin. Cut sections, mount without further staining after removal of paraffin.

Method III. Good for spinal cord, pons, medulla oblongata, spinal and sympathetic ganglia. Thin slices of tissues are fixed for five to six days in pure pyridine changed at least once, and then treated with repeatedly changed distilled water until the pyridine has been entirely eliminated. The surfaces of pieces are smoothed by means of a sharp razor, and the pieces brought for twenty-four hours into 4 per cent. ammonium molybdate to which 4 drops of hydrochloric acid have been added. After a quick wash, they are rapidly dehydrated in 95 per cent. and absolute alcohols, and imbedded in paraffin. The sections, which must be rather thin (3 to 6 μ), are brought through xylol, absolute and 95 per cent. alcohols into distilled water and here washed.

This is the crucial point of the method because, by washing, ammonium molybdate becomes extracted from the sections, and the success of the subsequent staining depends almost entirely on carrying out the extraction up to the right point. The only

way of ensuring this consists in proceeding by trials, which must be repeated for every series of sections. Once the right amount of washing has been decided upon, one can proceed to stain even many slides at the same time by means of a 1 : 10,000 solution of thionine, to be freshly prepared every time from a less diluted stock solution.

The staining is a " progressive " one, and must be controlled under the microscope. It generally takes about twenty minutes to obtain it, at the end of which time the grey substance has a red-purple tone whilst the white substance is bluish. If the staining is right the preparations can be quickly washed, dehydrated and mounted. But if the neurofibrils are not quite sharply stained, the preparations can be " differentiated " for another fifteen to twenty minutes in the ammonium molybdate solution used for mordanting the pieces, or for ten seconds in a diluted solution (1 : 10 to 1 : 20) of " pink salt " (*C. Erba, Milano*). Preparations last only a few months, but are sometimes of great interest. See DA FANO, *Ziegler's Beiträge*, xliv, 1908, p. 495.

Method IV is particularly useful for the demonstration of neurofibrils in the cells of the cortex cerebri and cerebelli. It differs from Method III only as regards a preliminary fixation of the pieces for twenty-four hours in a mixture of pyridine nitrate 10 grm., and pyridine, 100 c.c.; they are then transferred for another thirty-six hours into pure pyridine, proceeding as in Method III.

Method V may be used for the demonstration of both Nissl's substance and neurofibrils. Pieces are fixed in a saturated solution of corrosive sublimate; after a day they are treated for twenty-four hours with distilled water to which a few drops of iodine tincture have been added, then for two to three hours with pure distilled water; and lastly passed for forty-eight hours into pure pyridine, this being changed at least once. The rest as in Method III.

Method VII (for pericellular investments). Fix small pieces of tissue in a saturated solution of corrosive sublimate for twenty-four hours. Remove excess of sublimate by means of tincture of iodine, 15 drops in 100 c.c. of water. Wash in distilled water two to three hours. Place in pyridin, changing once, for thirty-four to forty-eight hours. Remove pyridin by washing for twenty-four hours in distilled water, repeatedly changed. Place in 4 per cent. ammonium molybdate containing 4 drops of pure hydrochloric acid for every 100 c.c. Wash in water for half to one hour. Return the pieces to fresh pyridin, changing this once, for thirty-six to forty-eight hours. Stain in 1 in 10,000 or 1 in 15,000 thionin for forty-eight hours, suspending them in the solution as in Method I. Transfer the pieces, still attached to the cork into the same acid solution of ammonium molybdate and

leave for twenty-four hours. Wash in repeatedly-changed distilled water for twenty-four hours, pass through alcohols and xylol into paraffin, and cut sections. Mount, after removing paraffin, without further staining.

PARAVICINI (*Boll. Mus. Z. Anat. Comp. Torino*, xx, 1905, p. 1) fixes and mordants in the dark, and differentiates after staining with extremely weak hydrochloric acid.

TOMASELLI (*Ztschr. wiss. Mikr.*, xxiii, 1906, p. 421) fixes spinal ganglia for six to seven hours in absolute alcohol 100 c.c. with 4 to 5 drops of ammonia, and then transfers them for two days into pure pyridine to be repeatedly changed, the vessel with the pieces being kept at 36° to 37° C. After washing for two to three hours in running tap-water, he continues as in Donaggio's Method III.

For the cricticism of JÄDERHOLM, see *Arch. mikr. Anat.*, lxvii, 1906, p. 108 ; and for that of MONTANARI, *Ztschr. wiss. Mikr.*, xxviii, 1911, p. 22.

1011. RAMÓN Y CAJAL'S **Methods. Introductory.** It has been said by some authors that Cajal's methods were originally only modifications of the photographic process of Simarro. The criticism is unjust because even the first formula of Cajal differs so profoundly from Simarro's process as to form an entirely new method. One cannot, however, deny the existence of a certain similarity of conception between the two processes in so far as both are based on the silver-reducing power of certain photographic reagents. For this reason it has been thought expedient briefly to describe here Simarro's process, which though uncertain in its results, may still be of some value to elucidate certain histological questions.

SIMARRO'S **Process** (*Rev. Trim. Micr.*, v, 1900, p. 45) consisted in poisoning animals with subcutaneous injections of solutions of sodium or potassium bromide or iodide in order to impregnate their living nervous tissues with one or the other of these salts. As soon as the animals showed that the poisoning had reached its maximum they were killed and their central nervous system removed in the photographic dark room. Small pieces were then immersed in a solution of silver nitrate, which, by combining with the bromine or iodine with which the tissues were impregnated, gave rise to the formation of silver bromide or iodide, which is easily affected by light. Sections were then made (always in the dark room), best by means of a freezing microtome, and exposed for a little while to light. There remained only treating them with a photographic developer, such as hydroquinone, pyrogallol or the like, and fixing them with sodium hyposulphite and so on, as if they were photographic plates ; they were lastly washed, dehydrated and mounted in the usual way.

One can easily understand the many drawbacks of such a method and the reason for which it was abandoned as soon as Cajal published in 1903 his first reduced silver methods. From that time onwards, Ramón y Cajal continued improving them and adding new formulæ, which he himself summarised in a special article of his *Trab. Lab. Invest. Biol.*, Madrid, viii, 1910, on which the following account is based. The numbering is that of Ramón y Cajal.

Formula 1. Small pieces of fresh tissue are put directly into 1·5 per cent. silver nitrate and kept therein for three to four and even to five days at a temperature of about 35° C. In summer, with a temperature constantly over 22° C., the stove may be dispensed with, provided the impregnation is prolonged for two to three days longer. The tissues are known to be ripe for reduction when a freshly cut surface shows a brownish-yellow colour. In this country it is better to use incubator temperature.

They are then washed for one to two minutes in distilled water and put into—

Pyrogallol or hydroquinone . .	1—2 grm.
Distilled water	100 c.c.
Formalin	5—10 c.c.

The formol is not necessary but useful. One may use pyridine instead (1 to 3 per cent.). The addition of a small quantity of sodium sulphite (0·2 to 0·5 per cent.) has been abandoned by Cajal. The stronger the pyrogallol or hydroquinone solution, the greater the contrast, so that it may be useful to take, sometimes, as much as 3 per cent. of either the one or the other, but then the over-impregnation of the outer layers is increased. Hydroquinone reduces more energetically than pyrogallol.

The pieces remain in the reducing fluid for about twenty-four hours and are then quickly washed, hardened in alcohol and imbedded in paraffin or celloidin. The sections (15 to 20 μ thick) are mounted in dammar after toning with a solution of gold chloride if the reaction is rather weak, without toning if the impregnation is a good one.

Faintly impregnated sections can be advantageously toned with—

Distilled water	100 c.c.
Ammonium sulphocyanide . .	3 grm.
Sodium hyposulphite . . .	3 ,,
1 per cent. gold chloride . .	Some drops.

If subsequently found to be too dark they can be bleached by Veratti's potassium permanganate and sulphuric acid mixture (see § 722).

The sections from the outer layer are generally too dark for study, those from the innermost too pale, whilst those from the intermediate layer are good. The over-staining of the outer layer can be diminished by diluting the silver nitrate with 1 volume of water for the last twelve hours.

The method has the defect of giving an imperfect fixation of the nervous tissue and of impregnating, almost exclusively, cell bodies and dendrites. It is not good for ganglia and large cells of adult subjects, but excellent for small and medium-sized cells of very young subjects and early embryos.

Formula 1, A. As the last, but pieces are fixed in 3 to 6 per cent. silver nitrate. This formula gives better fixation, and was successfully used by Dogiel (*Anat. Anz.*, xxv, 1904, p. 558, and *Arch. mikr. Anat.*, lxvii, 1906, p. 638) for the study of Grandry's corpuscles and other sensory nerve-endings, by Kolmer (*Anat. Anz.*, xxvi, 1905, p. 560) for the epidermis of *Lumbricus*, etc., and by other authors for the ganglionic chain of Hirudinea.

Formula 1, B. As above, but taking 0·75 per cent. silver nitrate and very small pieces, preferably from embryos and new-born subjects. Poor fixation, much shrinkage, but vigorous stain of the neurofibrils, nucleolar granules and the *intranuclear rodlet of Roncoroni.*

Formula 1, C. As above, but tissues are fixed in 2 per cent. silver nitrate to which one-fourth of absolute alcohol or acetone has been added. Better fixation than with pure silver nitrate. Results very similar to those obtainable by Formula 1 with dog, cat and rabbit, and better results with human cerebrum and cerebellum.

Formula 2. Fixation for twenty-four hours in 96 per cent. alcohol. Tissues not washed, but mopped with blotting paper and put into 1·5 per cent. silver nitrate for seven days at 35° C., or six days at 40° C. The rest as Formula 1. Good impregnations of nerve-centres of adults, of peripheral nerve-endings, of regenerating nerves, of nerve-cells of early embryos, and of young fishes. It impregnates medullated and many non-medullated fibres, large and medium nerve-cells, the basket fibres of Purkinje's cells, etc. Results fairly constant, but sometimes showing a granular precipitate of unknown origin.

To hinder this precipitate and, at the same time, to hasten the impregnation, it is well to add to the alcohol certain substances which Cajal calls *accelerators*. Such are chloral hydrate, veronal, pyridine, nicotine, ethylamine, antipyrine, and others.

Hypnotics, particularly veronal and chloral, and in a less degree pyridine and ammonia, also act as *rejuvenators*, reviving the susceptibility of impregnation in tissues which have lain too long in alcohol.

Formula 2, A. Fixation for twenty-four to forty-eight hours in 96 per cent. alcohol with 2 per cent. of chloral hydrate. Silver bath of 1·5 per cent. for five days in the stove. The rest as usual. Veronal (same proportion) gives the same result, as do also sulphonal, trional, hedonal, etc. The results are very constant. Medullated fibres well shown.

Formula 2, B. Fix for twenty-four hours in 96 per cent. alcohol with 10 to 20 per cent. of pyridine; wash for some hours in pure alcohol and transfer pieces into 1·5 silver nitrate for five days at 37° C.

This formula may be successfully employed for the study of

peripheral nerve-endings. In this case material is better fixed for twenty-four hours in pyridin to which one-third its volume of distilled water or 96 per cent. alcohol has been added. Pieces should be washed in running tap-water overnight and then transferred for six hours into pure 96 per cent. alcohol. Impregnation, reduction, imbedding, etc., as above. Results are good, but pieces become extremely hard even if dehydrated very quickly, and are consequently difficult to cut. See also Formula 5.

Formula 2, C. Fix for twenty-four hours in 50 c.c. of alcohol with 10 drops of nicotine. Mop up with blotting paper, without washing, and silver as usual for five days (or four at 40° C.). Good results with adult tissues, especially spinal cord. Good penetration and less shrinkage than with pure alcohol.

Formula 2, D. Fix for twenty-four hours in *allylalcohol* (the industrial product will do). Wash for some hours in several changes of water. Put for a day into 50 c.c. of alcohol with 4 drops of ammonia. Silver for four days at 35° to 38° C., and reduce as usual. Good for human tissues, especially for fibre plexuses of cerebrum and cerebellum. Instead of allylalcohol one may take acetal or acetone. Put for six hours into acetone with 25 per cent. of water, then for twenty-four into pure acetone, wash in water, etc., as above.

Formula 3. Fixation in ammoniacal alcohol for twenty to forty-eight hours. The most generally useful formula is 50 c.c. of 96 per cent. alcohol with 4 to 5 drops of ammonia (of 22° strength). But for cerebrum not more than 1 to 3 drops; for cerebellum, ganglia, spinal cord and regenerating tracts, 4 drops; for neurofibrils of the large nerve-cells of the medulla oblongata and spinal cord, 9 to 10 drops. To avoid shrinkage, it is well to begin by putting the pieces for six hours into 70 per cent. alcohol, then in 85 per cent., without ammonia; then for the rest of the time into the ammoniacal alcohol. Do not wash, but mop up with blotting paper before putting into the silver. Silver for four to four and a half days (small specimens) at 40° C., or medium to large (3 to 4 mm. thick) for five days at 32° to 35° C. So long as the tissues are only yellowish-white, they are not ripe for reduction; light grey indicates ripeness; dark grey over-ripeness. Reduce as by Formula 1.

Specimens may be decalcified, after reducing and washing, in 96 per cent. alcohol to which a few drops of nitric acid have been added.

For the impregnation of the neurofibrils of large and medium nerve-cells this formula is superior to all others. It gives good results with the majority of nerve-centres, and is particularly good for non-medullated fibres, peri-cellular baskets of cerebellum, buds of Held and Auerbach in the oblongata, spinal and sympathetic ganglia and regenerating nerve-fibres.

Formula 3, A. Fix in 50 c.c. of alcohol with 10 grm. of glycerin and 6 to 10 drops of ammonia. Good for retina and non-medullated fibres, but especially for the buds of Held and Auerbach.

Formula 3, B. Fix in 50 c.c. of alcohol with 1·5 c.c. of a 33 per cent. alcoholic solution of *ethylamin*. Results the same as with ammoniacal alcohol.

Formula 4. Pieces of tissue of not more than 4 mm. in thickness are fixed for six to twelve hours in 15 per cent. formol. Wash for six or more hours in running tap-water. Put for twenty-four hours into 50 c.c. of alcohol with 5 drops of ammonia. Wipe with blotting paper, silver for five days (or four if the stove is at 38° to 40° C.). The rest as usual. Sharp impregnation of the finer fibres of nerve-centres and of the terminal buds of pericellular nests. Adult tissues give better results than young ones. Energetic stain of the arborisations of the moss fibres of the cerebellum.

Formula 4, A. Fix in a mixture of formol and alcohol. Wash out thoroughly with running tap-water, silver, and reduce as usual. Fixation more rapid and better; results similar to those of 3.

Formula 5. This is characterised by a preliminary fixation in pyridin as originally suggested by HELD (*Arch. Anat. Physiol., Anat. Abth.*, 1905, p. 77; *Anat. Anz.*, xxix, 1906, p. 186). He used to fix tissues in pure pyridin, but Cajal finds that this is likely to cause much shrinkage, and he recommends fixing small pieces first for six to eight hours in a mixture of equal parts of distilled water and pyridin, then for eighteen to twenty-four hours in pure pyridin. Wash for several hours in running water, and put for a day into 90 per cent. alcohol. Wipe, and put for four to five days into 1·5 per cent. silver nitrate at 35° to 38° C., and reduce as usual. Not very good for adult organs, but superior to all others for the earliest phases of neurogenesis, and good for regenerative processes, as well as for peripheral nerve-endings.

Formula 6. Put for twenty-four hours into 50 c.c. of water with 5 grm. of *chloral hydrate*, rinse, and put into 50 c.c. of 96 per cent. alcohol with 5 drops of ammonia (time not stated). Wipe with blotting paper; put for four to five days at 35° to 38° C. into 1·5 per cent. silver nitrate, and reduce as usual. Results very constant, without shrinkage. Good for the fine plexuses of cerebrum, bulb and cord, the baskets of Purkinje's cells, and moss fibres; also for motor plates and for regenerating nerves.

Denny-Brown and Hoff have used this method with success for staining the "boutons terminaux" on the anterior horn cells of the spinal cord. In experimental work they inject 10 per cent. chloral hydrate into the aorta after washing out the vascular system with saline.

Formula 6, A. Fix for twenty-four hours in 10 per cent. *chloral hydrate*, wash for six hours and put direct into the silver. Stove for four days. Results similar to those of Formula 1. Medullated fibres well stained.

Formula 7. Fix for twenty-four hours in Merck's *fibrolysine*, wash for six, put for twenty-four into 50 c.c. of alcohol with 5 drops of ammonia. The rest as by other formulæ.

Instead of fibrolysin, *lysidine* may be taken.

1012. Application of Cajal's Methods to different Objects. 1. For the study of the evolution of neuroblasts and nerve-fibres in *very early embryos* it is necessary to avoid fixing with formol, or alcohol with an accelerator, or ammoniacal liquids. The best formulæ are 2 and 5 which are applicable to all vertebrates, but preferably to embryos of birds and fishes.

2. For *late embryos and fœtus* of mammals. Besides the above formulæ, 3, 6 and alcohol with an accelerator. Best subjects, embryos of chick from the fifth day, and of rabbit from the tenth to the twelfth day; or new-born birds, with ammoniacal alcohol, or 5.

3. For *sympathetic ganglia.* Formula 3, or pure alcohol, or 4 and 5. Best with man. Dog, cat, and rabbit give mostly weak reactions. The visceral ganglia are the most difficult.

4. *Sensory ganglia.* Formula 2 or 3. Easy.

5. *Cerebellum.* For Purkinje cells, 1 or 3. For the baskets, climbing fibres, and medium and small dendrites, 2 or its variants. For terminal rosettes and collaterals of moss fibres and for the plexuses of the granular layer, 4 or sometimes 5 or 6. For the stellate cells of the molecule layer, 2 and 3. The best subject for the latter is the dog.

6. *Cerebrum.* In general, the same formulæ as for the cerebellum, especially 1 for pyramids of young dogs and cats (of eight to twenty days). In Formula 3 the proportion of ammonia should be diminished. For fine plexuses, 4, 5, and 6.

7. *Spinal cord and bulb.* All the formulæ are applicable. For neurofibrils of motor cells the best subject is the dog of four to fifteen days, with Formula 3, with a large dose of ammonia (10 drops); also the alcoholic fixatives with an accelerator. For medullated fibres, large and small, 2 or 6. For buds of Held and Auerbach and for fine plexuses, 4, 3, A, or 5.

8. *Ganglia of invertebrates.* For the medicinal leech (not for other leeches), 1, A. For *Hæmopis, Aulostomum, Pontobdella* and *Glossiphonia*, 2 or, better, 3, with not more than 2 to 5 drops of ammonia and 3 per cent. silver nitrate, stoving three or three and a half days. For further details see Sanchez, *Trab. Lab. Invest. Biol.*, Madrid, vii, 1909, pp. 42—47.

Lumbricus is generally refractory to Cajal's methods. Boule (*Le Nevraxe*, x, 1908, p. 15) obtained good impregnations by

acidifying the fixatives. He takes: (*a*) 25 per cent. formol with 5 per cent. of acetic acid; or (*b*) the same with 0·5 per cent. of ammonia; or (*c*) 100 c.c. of alcohol, 25 c.c. of formol, 5 c.c. of acetic acid, and 0·5 c.c. of ammonia. For the impregnation he uses 3 per cent. silver nitrate with 15 per cent. of alcohol, and reduces in the usual hydroquinone-formol solution, with the addition of 15 c.c. of alcohol. These results are confirmed by KOWALSKI (*La Cellule*, xxv, 1909, p. 292, and by SZÜTS (*Anat. Anz.*, xlii, 1912, p. 262). Kowalski gets impregnations also by simply starving worms for several days, or exposing them to cold (—5° C.) for a quarter of an hour.

9. *Regenerating nerve-tissue.* For nerves operated on a month or more previously, Formula 2 or 3, with not more than 3 drops of ammonia, will stain equally the old and the new fibres; for nerves operated on not more than two to ten days previously, Formulæ 3 with 4 to 6 drops of ammonia, 5 with pyridin, and 4, also sometimes 6; for regeneration in cord, cerebrum, and cerebellum, 3 with 3 drops of ammonia, or 5, or pure alcohol.

1013. Modifications of RAMÓN Y CAJAL'S Methods. DA FANO (*Ziegler's Beitr.*, xliv, 1908, p. 495) recommends using solutions of silver nitrate and hydroquinone in 1 : 10,000 gelatin in order to obtain a deeper and sharper impregnation.

KATÓ (*Folia neurobiol.*, ii, 1908, p. 262) fixes in 10 to 15 per cent. formol, and silvers for one to five days at 35° C. in 5 per cent. argentamin to which 3 per cent. of silver nitrate has been added in such a way as to have an impregnating fluid with a little argentamin in excess; or argentamin 8 to 10 parts, with 3 per cent. potassium bichromate 30 parts and distilled water 100 parts. For the reduction he uses 10 per cent. formol with 1 per cent. hydroquinone.

PUSATERI (see AMATO, *Virchow's Arch.*, clxxxv, 1908, p. 547) fixes for three to six days at 35° to 38° C. in a mixture of 45 c.c. of *tachiol* (10 per cent. silver fluoride) and 155 of distilled water.

BESTA (*Riv. pat. nerv. ment. Firenze*, xv, 1910, p. 333) fixes for forty-eight hours in alcohol with 5 per cent. nitric acid, neutralised in alcohol with ammonia.

LIESEGANG (*Kolloidchemie Beihefte*, iii, 1911, H. 1, p. i; *Ztschr. wiss. Mikr.*, xxviii, 1912, p. 369) makes sections of formol material by the freezing method, and puts them in 1 per cent. silver nitrate in the dark for hours or days until yellow. If necessary, increase the strength of the silver solution or place the dish in the incubator. Pour off the silver solution with the exception of about 2 c.c.; add equal parts of 50 per cent. gum and of a concentrated solution of hydrogen potassium sulphide. Wash in distilled water and mount in the usual way in balsam.

ASCOLI (*Boll. Soc. med. chir.*, Pavia, 1911, p. 177) recommends for the nervous system of Hirudinea the following: The animals cut open at the back are stretched on a piece of cork and fixed in a solution prepared by dissolving over a flame 5 gr. of pulverised crystals of silver nitrate in 100 c.c. of 95 per cent. alcohol. After a few minutes the animals may be detached from the cork and put back in the same fixative for twenty-four to forty-eight hours in an incubating stove. They are then transferred for another twenty-four to forty-eight hours into a 10 per cent. watery solution of silver nitrate, to be kept also in

the incubator. After a quick wash they are reduced for five to eight hours in Amidol-Hauff 0·5 gr., sodium sulphite cryst. 10 grm., distilled water 100 c.c., and lastly passed into glycerin. Preparations are made by teasing, the thinner ones being toned and counterstained as usual. For mounting he prefers Apáthy's syrup.

RANSON (*Anat. Anz.*, xlvi, 1914, p. 522) has the following for the demonstration of non-medullated nerve-fibres in cranial and peripheral nerves: Fix in absolute alcohol containing 1 per cent. of strong ammonia for forty-eight hours; rinse in distilled water, put in pyridine for twenty-four hours, place in 2 per cent. silver nitrate at 35° C. in the dark for three days, rinse in water, and place for one day in a 4 per cent. solution of pyrogallic acid in 5 per cent. formalin. HEWER (*Journ. Anat.*, lxvii, 1933, p. 350) uses Ranson's technique on material fixed in 7 per cent. formalin and has had satisfactory results by reducing in 4 per cent. pyrogallol without the addition of formalin.

As suggested by HÜBER and GUILD (*Anat. Rec.*, vii, 1913, p. 253) the results can be improved by a preliminary injection of 95 per cent. alcohol, containing 1 per cent. of ammonia, through the arteries till tissues are thoroughly saturated, after which they are dissected out and placed in a similar ammoniated alcohol solution for from two to three days. Hüber and Guild have found this method of use for the study of cranial nerves of small animals and embryos, since the entire heads can, after fixation, be decalcified by means of 7 per cent. nitric acid, brought through 80, 90, and 95 per cent. alcohols, each containing 1 per cent. of ammonia, and finally treated as above.

C. RECENT METHODS

1014. RAMÓN Y CAJAL'S **(1) Method for Sections of Cortex Cerebelli** (*Trab. Lab. Invest. Biol.*, xix, 1921, p. 71). The idea of this method appears to have been suggested in part by Liesegang's modification (*op. cit.*, p. 1057) in part by the principle underlying the Bielschowsky method for sections. Frozen sections from formalin material are collected in water to which a few drops of formalin are added. Before carrying out the staining they are washed in two changes of distilled water and transferred into a bath consisting of 10 c.c. of 2 per cent. silver nitrate and 5 to 8 drops of pyridin. They are either left therein in the dark from twelve to forty-eight hours or (*Trab. Lab. Invest. Biol.*, xxiii, 1925—6, pp. 162—4) warmed over a flame for some minutes. When they have assumed a light brown colour they are placed for half a minute in 96 per cent. alcohol to which 2 to 3 drops of 2 per cent. silver nitrate may be added if an intense stain is desired. Without washing, the sections are transferred into the reducing fluid, consisting of 30 c.c. of formalin, 70 c.c. of distilled water and 0·20 to 0·30 grm. of hydroquinone. The reduction is complete after one minute, when the sections can be washed in distilled water, dehydrated, cleared and mounted in balsam. Toning is not, as a rule, necessary; but it can be easily carried out by means of a 1 : 300 solution of gold chloride followed by fixation in dilute sodium hyposulphite or 1 per cent. thiosinamine. If the background is very dark the sections can

be "cleared" before dehydration in ½ per cent. potassium ferricyanide, followed by a wash in distilled water, and the usual treatment with sodium hyposulphite. The method can be used also for the study of the cerebral cortex where the fine (non-medullated) fibres of the grey layers are well impregnated. If it is desired to bring into view the axons of the large medullated fibres the sections can, after cutting, be placed for six hours in 96 per cent. alcohol or in a bath consisting of 4 grm. of iron alum, 100 c.c. of distilled water and 1 grm. of oxalic acid. A similar result can be obtained by adding to the silver-pyridin bath a quarter its volume of 96 per cent. alcohol.

1015. (2) Method for Central and Peripheral Nerve-endings (*Trab. Lab. Invest. Biol.*, xxiii, 1925—6, p. 237). Frozen sections (30—40 μ) from formalin material are collected in distilled water, washed and transferred for four to six hours into an impregnating bath consisting of 2 per cent. silver nitrate 10 c.c., pyridin 7 to 10 drops, 96 per cent. alcohol 5 to 6 c.c. They should assume a light brown colour, which can be intensified by placing the dish over a flame for a few minutes. The sections are then *quickly* washed (no more than two or three at a time) in 96 to 98 per cent. alcohol, and immediately afterwards reduced in a bath consisting of 0·30 grm. of hydroquinone, 70 c.c. of distilled water, 20 c.c. of formalin, 15 c.c. of acetone. After a few minutes they are washed, toned, fixed and mounted in the usual way.

1016. (3) Method for Pericellular Baskets, Moss and Climbing Fibres (*ibid.*, p. 240). This method can be considered as a simplification of the previous one. Frozen sections from formalin material are impregnated and reduced at the same time in a bath consisting of 10 c.c. of 2 per cent. silver nitrate, 10 drops of pyridin and 7 to 10 drops of formalin. The sections become dark brown in about four to six hours, when they are washed, toned, etc., as above. This method is not recommended either for the man or rabbit.

De Castro's Modifications. The following formulæ have recently been suggested by De Castro (*Trav. Lab. Rech. Biol.*, xxiii, 1925—6, p. 427) for fixing and decalcifying at the same time. Small pieces can be fixed by means of one or the other of the following mixtures.

1.	Chloral hydrate	2·5 grm.
	Distilled water	50 c.c.
	Alcohol (presumably 96 per cent.)	50 ,,
	Nitric acid	3 to 4 c.c.
2.	Urethane (ethylic)	1–2 grm.
	Distilled water	40 c.c.
	Alcohol (presumably 96 per cent.)	60 ,,
	Nitric acid	3 to 4 c.c.

3.	Somnifene (Hoffmann—La Roche) .	2 to 4 c.c.
	Alcohol (presumably 96 per cent.) .	60 c.c.
	Distilled water	40 ,,
	Nitric acid	3 to 4 c.c.

After one to three days decalcification is complete, particularly if the pieces are small and from very young or fœtal mammals. They are washed in distilled water for twenty-four to thirty-six hours to extract the nitric acid and then transferred into alcohol, 96 per cent. containing 4 to 6 drops of ammonia for every 50 c.c. of alcohol. After twenty-four hours they are silvered and reduced as by Cajal's method. Some pieces can, after washing, be dehydrated and imbedded either in celloidin or paraffin and the sections stained by either the Nissl method or ordinary methods.

1017. Bielschowsky's **Methods. Introductory.** It is well known that, if ammonia be poured into a solution of silver nitrate, a precipitate is formed which is re-dissolved by the addition of some more ammonia. If an alkaline solution of formaldehyde be slowly added to this easily reducible diammoniacal silver nitrate ($N(NH_4)AgH_2NO_3$), metallic silver is immediately precipitated and deposited on the walls of the test-tube. Both Fajerstajn (*Neurol. Centrbl.*, xx, 1901, p. 98) and Bielschowsky (*ibid.*, xxi, 1902, p. 579) thought of taking advantage of this reaction for histological purposes with the object of finding out a silver impregnation of the nervous tissue similar to that which characterises Golgi's method. The results of their attempts were different: Fajerstajn was able to obtain only a difficult method for staining axis-cylinders which is now superseded; Bielschowsky also published, at first, a complicated silver method for impregnating axis-cylinders very similar to that of Fajerstajn, but, through successive modifications of his first process, was led to the discovery of a new method, which is as important as Cajal's reduced silver methods from an histological point of view, but is of still greater advantage than the latter for histopathological investigations. Moreover, Bielschowsky's method is applicable to any formol material, even if very old. Bayon (*Die Untersuchungsmeth*, etc.) succeeded with four-year-old material, and Da Fano with brains which had been left in formalin for more than eleven years.

There are at present *three* Bielschowsky methods: one for sections, one for peripheral nerve-fibres and axis-cylinders, and one for pieces. It seems better to describe them separately in the following account which is based on the original papers of Bielschowsky, as well as on some personal experience Da Fano gained through a visit paid to him when in Berlin.

1018. Bielschowsky's **Method for Sections** (*Journ. Psychol. Neurol.*, iii, 1904, p. 169; and xii, 1909, p. 135). Pieces from

central nervous organs, thoroughly fixed in 15 to 20 per cent. formalin, are washed for some hours in running tap-water and then cut by means of a CO_2 freezing microtome. The sections are collected in distilled water, thoroughly washed therein and passed into a 2 or 3 per cent. solution of silver nitrate where they are left for twenty-four hours in a dark place, and at room temperature. The sections can also be passed *first into pure pyridine for twenty-four to forty-eight hours*, washed in many changes of distilled water until the pyridine has been completely eliminated and then transferred into 2 or 3 per cent. silver nitrate as above.

The pyridine bath is optional and has the advantage of ensuring a sharper stain of axis-cylinders whilst neuroglia, which is more or less coloured when the pyridine bath is dispensed with, remains unstained. Also connective tissue and nuclei are generally very faintly stained after the pyridine treatment. Intracellular neurofibrils, however, are *not always* so well shown as by the direct passage of sections into the silver nitrate solution.

Before proceeding further, one should prepare the Bielschowsky ammoniacal silver nitrate-and-oxide bath as follows: Pour 5 c.c. of a 20 per cent. solution of silver nitrate into a measuring cylinder and add to it first 5 drops of a 40 per cent. solution of NaOH, and then ammonia, drop by drop, until the brown precipitate formed disappears; dilute to 25 c.c. with distilled water, and filter through paper washed with the same water.

For staining, take sections one by one from the silver nitrate bath, quickly wash them in distilled water for not more than a few seconds, and transfer them into the ammoniacal silver bath. Here they remain for about ten minutes when they become yellowish-brown and should be, once more, quickly washed in distilled water and placed in 20 per cent. formalin prepared with tap water. The reduction takes place immediately, and if one works with a number of sections it is advisable to re-transfer them into a fresh bath of 20 per cent. formalin.

At the end of half an hour and even less, the reduction can be considered as accomplished and sections can be washed in distilled water and toned with a diluted (0·2 per cent.) solution of gold chloride. This may be slightly acidified with acetic acid if one wishes to obtain a faintly purple background, or neutralised with a few drops of a diluted solution of sodium or lithium carbonate if one prefers greyish-white backgrounds. Instead of gold chloride one can use a slightly acid solution of chloroplatinic acid. After toning there remains only once more to wash the sections in distilled water, and to pass them for a few minutes into a 5 per cent. solution of sodium hyposulphite, or any diluted fixing bath for photographic plates. Wash again, dehydrate in alcohols of increasing strength up to 95 per cent., clear in carbol-xylol, and mount in balsam.

For other details about the toning and fixing of sections see the original papers of Bielschowsky (*op. cit.* and *Journ. Psychol. Neurol.*, iv, 1904–5, p. 227), as well as Wolff (*Biol. Centrbl.*, xxv, 1905, p. 683), and Da Fano (*Proc. Physiol. Soc. Journ. Physiol.*, liii, 1920).

Bielschowsky states that this method is also suitable for sections of celloidin or paraffin blocks of formol material, but he does not recommend the practice, and we have no experience of it.

1019. Bielschowsky's **Method for Peripheral Nerve-fibres** (*Journ. Psychol. Neurol.*, iv, 1904–05, p. 227). This method can be applied to the study of spinal and sympathetic ganglia, peripheral nerve-endings, and end-organs in normal conditions, but its chief applications belong to the domain of histopathology. According to our experience good results are rarely obtained, and the method requires important modifications to become as useful as the above and following ones.

The staining is carried out on sections of formol material in the same way as described above. There is only this difference that the staining in the ammoniacal silver bath is carried on a few minutes longer, viz., until the sections have taken a decidedly brown colour, after which they are washed in 10 c.c. of distilled water acidified with 5 drops of acetic acid, when they acquire (sometimes in a few seconds) a yellowish tinge. They should then be immediately transferred into the usual 20 per cent. solution of formalin. For the toning a neutral gold bath is necessary; sections should be left therein until red-violet. In the finished preparations axis-cylinders are black, myelin red-violet, connective tissue violet or blue-violet. The washing in acidified water and the prolonged toning both answer for the purpose of creating a sharp contrast between nerve-fibres and connective tissue-fibres, which might otherwise become stained almost as black as the axis-cylinders.

I have found that counterstaining the sections for from 10 to 20 seconds in van Gieson's mixture before mounting serves to differentiate as well as to stain the connective tissue-fibres, so that they are not so easily confused with the nerve-fibres. (J. G. G.)

Bielschowsky has also a method for *central* nerve-fibres. Sections made by freezing from formol material are placed for twenty-four hours or longer in a 4 per cent. solution of copper sulphate or Weigert's mordant for neuroglia stain (§ 1083). After washing they are placed for a few seconds in the usual ammoniacal silver bath and then washed, reduced, toned and fixed as above. The preparations are similar to those obtainable by the methods of Fajerstajn, Strahüber and Kaplan.

1020. Bielschowsky's **Method for Pieces** (*op. cit.*). Good for peripheral nerve-endings and embryonic material, and also for

small specimens of adult subjects. This method has been described by Bielschowsky in various ways, probably because of the difficulty of giving fixed rules in a case in which the greatest freedom had to be left to histologists to adapt the method to the quality of their material and the purpose of their investigations. In what follows two forms of the method are described: one without and one with pyridine treatment of pieces.

A. *Method for Pieces without Pyridine Treatment.* Thin slices or small pieces of formol material are washed for some hours, first in running tap-water and afterwards in distilled water. They are then placed in a 2 per cent. solution of silver nitrate for from one to eight days in the dark. The use of an incubator at 35° to 37° C. is optional. After a wash in several changes of distilled water (to be prolonged for some minutes up to some hours according to the length of time during which pieces have been kept in the silver bath, and if in an incubator or not) they are transferred into an ammoniacal solution of silver nitrate prepared as in the method for sections, but diluted up to 100 c.c. They are kept therein for from an hour up to six, washed once more in distilled water, passed for twelve to twenty-four hours into the usual 20 per cent. solution of formalin. Wash, dehydrate quickly, imbed, preferably in paraffin, tone sections as described above, counterstain, if necessary, mount in balsam.

B. *Method for Pieces with Pyridine Treatment.* Pieces of formol material, up to 1 cm. thick for adult tissue, and up to 5 cm. long for embryos, are put for two, three or four days into pure pyridine, washed for some hours in several changes of distilled water and put for three to five days into 3 per cent. silver nitrate at 36° C. Wash in distilled water and transfer into the diluted ammoniacal silver bath as above, but leaving pieces therein for twenty-four hours. Wash for about two hours in several changes of distilled water, reduce in 20 per cent. formalin. The rest as above.

1021. Method for Membranous Labyrinth of Mammals. 1. Fix entire temporal bone in 20 per cent. formalin with 5 per cent. nitric acid, and decalcify completely in this. Return to 20 per cent. formalin.

2. Make frozen sections, wash and place in 4 per cent. silver nitrate for twenty-four hours in the dark.

3. Wash rapidly and put sections for a few minutes until they have a brownish tone in an ammoniacal silver nitrate solution made as follows: To 5 c.c. of 20 per cent. silver nitrate add 5 drops of 40 per cent. caustic soda; add ammonia to dissolve the precipitate, and add 20 c.c. of distilled water.

4. Wash sections in slightly acidulated water (1 drop of acetic acid to 20 c.c. of distilled water).

5. Reduce in 20 per cent. formalin.

If necessary the operations described in (3) to (5) can be repeated once more after a thorough wash of the sections.

1022. Modifications of Bielschowsky's Methods. Favorsky (*Journ. Psychol. Neurol.*, vi, 1906, p. 260) uses 10 per cent. silver nitrate for the first silver bath instead of 2 or 3 per cent.

Paton (*Mitth. Zool. Stat. Neapel*, xviii, 1907, p. 576) fixes fish embryos in 4 per cent. formaldehyde neutralised with carbonate of magnesia. For the first silver bath he uses 0·75 to 1 per cent. silver nitrate and keeps material therein four days in summer, five to seven in cooler weather. To make the ammoniacal silver nitrate-and-oxide bath he takes 20 c.c. of 0·75 to 1 per cent. silver nitrate, adds to it 4 drops of 40 per cent. caustic soda and then ammonia drop by drop in the usual way. The embryos are first washed in distilled water, then kept for five to fifteen minutes in 10 c.c. of water acidified with 5 drops of acetic acid, washed once more in pure water, and transferred for twelve hours into a reducing fluid consisting of 1 per cent. hydroquinone 20 c.c., neutralised formalin 2 c.c. After imbedding in paraffin, the sections are toned as usual and counterstained with 1 per cent. eosin in absolute alcohol.

Schütz (*Neurol. Centrbl.*, xxvii., 1908, p. 909) finds that the times given by Bielschowsky are too short and washes sections for twenty-four hours after the 2 per cent. silver nitrate bath, leaves them thirty to forty minutes in the ammoniacal silver bath, and twenty-four hours in the 20 per cent. formalin. For toning he puts them for ten minutes into 10 c.c. of water with 2 drops of acetic acid, then for thirty to forty-five minutes into 10 c.c. of water with 3 drops of a 1 per cent. gold chloride solution (until blackish-grey).

Boeke (*Anat. Anz.*, xxxv, 1910, p. 193) has obtained excellent results by the use of Bielschowsky's method for pieces when applied to the study of peripheral nerve-endings. He fixes in 10 per cent. formalin prepared with 60 per cent. alcohol, changes the fluid two or three times, and then either leaves material therein until wanted or keeps it in 70 to 80 per cent. alcohol. For staining, pieces are brought into 10 to 12 per cent. formalin, and left in it until they are quite free from alcohol.

After washing, place in 2 per cent. silver nitrate for three to five days in the dark. Wash in distilled water rapidly, and place in Bielschowsky's silver oxide solution for one to two hours in the dark. Wash rapidly and reduce in 20 per cent. formalin. Imbed blocks in paraffin and cut sections.

Boeke finds that the method succeeds also after other kinds of fixation.

Schlemmer (*Ztschr. wiss. Mikr.*, xxvii, 1910, p. 22) makes the ammoniacal silver nitrate-and-oxide bath by adding to any silver nitrate solution, 40 per cent. caustic soda, drop by drop, until no more precipitate is formed. He then washes the precipitate by repeated decantation until the wash-water no longer gives an alkaline reaction, takes it up with the smallest possible quantity of ammonia, and filters through glass-wool. This concentrated solution keeps for many days unaltered, and should be diluted ten times its volume before using it.

Deikun (*Ztschr. wiss. Mikr.*, xliii, 1926, p. 380) gives the following directions for making up Bielschowsky's silver solution. All glassware must be thoroughly cleaned, and finally washed with distilled water. The solution must be freshly prepared at the moment of use; 2 c.c. of a 10 per cent. solution of $AgNO_3$ are poured into a test-tube, and 1 drop of 40 per cent. NaOH added to it. Shake gently and wait for the precipitate to fall to the bottom of the tube; add another drop of 40

per cent. NaOH, shake and again let the precipitate fall to the bottom. Repeat the operation a third and a fourth time till no precipitate is formed on adding NaOH. The supernatant clear fluid is now pipetted off and the precipitate washed with 100 to 150 c.c. of distilled water. Continue washing until the wash water no longer reddens phenolphthalein. Pipette off the wash-water and add ammonia drop by drop to the precipitate till it is almost but not entirely dissolved. After the addition of 4 c.c. of distilled water the fluid is ready for use.

Del Rio-Hortega (*Trab. Lab. Invest. Biol.*, Madrid, xiv, 1916, p. 181) has made known a similar method used in those laboratories for preparing the ammoniacal silver nitrate bath. Forty drops of 40 per cent. caustic soda are added to 30 c.c. of 10 per cent. silver nitrate, and the precipitate washed ten to twelve times by means of about a litre of distilled water. Fifty cubic centimetres of water are then added to it, and ammonia, drop by drop, until the precipitate is dissolved. The solution, brought finally to 150 c.c. and filtered into a dark brown bottle, keeps well for many months. I find that the ammoniacal silver bath thus prepared can be further diluted with one, two, up to five times its volume of water, and usefully employed for Bielschowsky's method for pieces, particularly for the study of peripheral nerve-endings.

Agduhr (*Ztschr. wiss. Mikr.*, xxxiv, 1917, pp. 1—99), who has exhaustively investigated almost all questions relating to the results obtainable by Bielschowsky's method for pieces, has come to the conclusion that material is best fixed in neutral or slightly acid 20 per cent. *formaldehyde* (50 per cent. formalin). Pieces should then be washed in distilled water for many days until the wash-water is free from substances reducible by an ammoniacal silver nitrate solution used as test. For the first silver bath he uses 3 per cent. silver nitrate, and for the second a solution obtained by adding to 10 c.c. of 10 per cent. silver nitrate, first 20 drops of 25 per cent. NaOH, then from 200 up to 600 c.c. of distilled water, and lastly ammonia enough to dissolve the precipitate. For the reduction he uses again 20 per cent. *formaldehyde*. To avoid an excessive impregnation of the connective tissue he also finds it useful to wash pieces in acidified distilled water (see the Bielschowsky's method for peripheral nerve-fibres), but he uses as much as five times the amount suggested by Bielschowsky.

1023. Da Fano's **Modifications.** In this series of modifications of Bielschowsky's method it is important that the distilled water should be pure and free from any trace of organic matter.

Da Fano's first modification (*Mod.* 1) (*Atti. Soc. Lamb. Sc. Med. Biol.*, Milano, iii, 1914) was meant for the study of reticular tissue of spleen, lymph glands, and other organs, and is to be carried out as follows: (1) Fix small pieces of fresh tissue in 10 to 20 per cent. formalin or in Kayserling's first fluid (forty-eight hours at least), or in Orth's fluid (twenty-four to forty-eight hours). (2) Wash pieces in running tap-water for twenty-four to thirty hours, and then in distilled water for another twenty-four hours. (3) Wash sections made by the freezing method in re-distilled water (twenty-four hours), and then place them in filtered 2 per cent. silver nitrate (prepared with re-distilled water) in a Petri dish, taking care that they do not touch each other. Here they are kept in the dark and at room tempera-

ture from six hours to three days. (4) Treat sections for twenty to thirty minutes with Bielschowsky's ammoniacal silver nitrate solution prepared with only 2 drops of 40 per cent. caustic soda and diluted with re-distilled water to 40 to 70 c.c. (5) Reduce, tone, counterstain, and mount as by Bielschowsky's method for sections.

Mod. 2 (*Proc. Physiol. Soc. Journ. Physiol.*, lii, 1919) consists in an application to nervous tissues of Mod. 1. The use of re-distilled water and the mode of preparing the ammoniacal silver bath are the same, but Da Fano lays stress on the following points: (1) Nervous tissue must be fixed in 10 up to 20 per cent. formalin for at least three weeks, better still for two months. Attempts to obtain a rapid fixation with 10 to 20 per cent. formalin at 37° C. gave bad results. (2) Sections of nervous tissues may be placed, after washing in re-distilled water, in anhydrous pyridine (six to twelve hours), then repeatedly washed and left overnight in re-distilled water, to get rid of all pyridine. This treatment appears to render neurofibrils a little thinner and, consequently, a little sharper, but increases the length and cost of the method, and may cause precipitates to form, especially where much myelin is present. (3) It is possible to keep sections, which cannot be stained immediately, for some days or even a fortnight, in re-distilled water to which a few drops of formalin have been added. Thorough washing with re-distilled water is then imperative before they are transferred into the 2 per cent. silver nitrate solution. (4) Sections of nervous tissues must not remain in the 2 per cent. silver nitrate more than forty-eight hours, or precipitates may form. The longer their stay there, the longer must be the washing before staining; this, however, must not, *as a rule*, exceed five minutes. (5) The volume to which the ammoniacal silver nitrate is diluted should be 35 to 45 c.c., and the sections remain in it fifteen to twenty minutes. The subsequent washing before transferring the sections into 20 per cent. formalin should not occupy more than ten to fifteen seconds, and their stay in the final formalin solution (especially for cerebral cortex) should not exceed two to three hours.

The other *eight* Da Fano modifications (*Proc. Physiol. Soc., Journ. Physiol.*, liii, 1919–20) were all proposed for the study of cortex cerebelli, and are characterised by a special treatment of the sections (cut by freezing method) with various reagents before transferring them into the 2 per cent. silver nitrate solution, nothing having been changed, however, in regard to the long fixation of material in formalin and the use of re-distilled water. They may be summarised as follows:—

Mod. 3. Place sections, after washing in re-distilled water, in 2 to 3 per cent. silver nitrate *at* 36° *to* 37° C. for about twenty-

four hours; wash quickly; stain in ammoniacal silver nitrate solution diluted to 40 c.c. for thirty minutes. Wash, reduce, tone, and mount as usual.

Mod. 4. Place sections in 50 per cent. pyridine for six to eighteen hours; wash in re-distilled water for twenty-four to forty-eight hours; 2 per cent. silver nitrate at 37° C. for twenty-four hours, etc., as in Mod. 3.

Mod. 5. Place sections in pure pyridine for four to twelve hours. Wash in re-distilled water overnight. Transfer sections into 20 per cent. formalin prepared with re-distilled water for about twenty-four hours. Wash again in re-distilled water overnight; 2 per cent. silver nitrate at 37° C., etc., as before.

Mod. 6. Sections are treated first with 20 per cent. formalin, and then with pure pyridine, in *the reverse order* of Mod. 5.

Mods. 7 *and* 8. The same as Mods. 5 and 6, but replacing the pyridine with a mixture of 3 parts of methyl alcohol and 2 parts of water.

Mod. 9. Place sections in a mixture of equal parts of 20 per cent. formalin and methyl alcohol for twenty-four hours; wash in re-distilled water for six to twenty-four hours; 2 per cent. silver nitrate at 37° C. for twenty-four hours, etc., as before.

Mod. 10. Place sections into 20 per cent. formalin for twenty-four hours, transfer them, *without washing*, into a mixture of equal parts of 20 per cent. formalin and methyl alcohol, etc., as in Mod. 9.

Mod. 3 is particularly suitable for human material of young individuals: Mod. 4 for adult subjects. Mods. 5 and 6 are useful for the study of neurofibrils in the various elements of the cortex cerebelli and for the staining of the granules. Mods. 7, 8 and 9 are to be preferred for the demonstration of pericellular baskets and nervous processes. Mod. 10 gives very complete stainings, and is the most certain of all; preparations are, however, fairly dark and, therefore, more suitable for general view.

1024. Neurofibrils; Other Methods. Cox's *Method* for fibrils of spinal ganglion cells; see *Ztschr. wiss. Mikr.*, xiii, 1896, p. 498, and *Anat. Hefte*, x, 1898, p. 98.

S. Meyer's *Berlin blue*, see *Anat. Anz.*, xx, 1902, p. 535.

Lugaro's *collargol* (colloidal silver) method, see *Monit. Zool. Ital.*, xv, 1904, p. 353.

Joris' *colloidal gold method* has not been received with favour; see *Bull. R. Acad. Med. Belg.*, xviii (S. iv), 1904, p. 293.

Sand (*C. R. Ass. Anat. Bruxelles*, 1910; *Bibliogr. Anat. Supp.*, 1910, p. 128, or *Ztschr. wiss. Mikr.*, xxviii, 1911, p. 500) gives the following as entirely certain for man, dog, cat, and rabbit. Specimens of not more than 5 mm. in thickness are fixed for forty-eight hours in a freshly-prepared mixture of 90 parts of acetone and 10 of nitric acid, to be changed for fresh after half an hour, and once again within twenty-four hours. Wash out for at least six hours in pure acetone, changed two or three times. Make paraffin sections and bring them

through xylol and acetone into distilled water; silver for three days at about 37° C. in 20 per cent. solution of silver nitrate. Put for ten minutes into a mixture (at least three days old) of 1000 parts of water, 10 of sodium acetate, 5 of gallic acid, and 3 of tannin (to be changed if it becomes turbid). Mount at once or tone until grey (five minutes) in 80 parts of water with 17 of 2 per cent. ammonium sulphocyanide and 3 of 2 per cent. gold chloride; fix for a few seconds in 5 per cent. sodium hyposulphite. Neurofibrils grey-violet, shown in cells, dendrites, and axons. Terminal buds of Held also clearly shown, and nothing else stained. One may counterstain in any way, even by Weigert's or Benda's methods for neuroglia stain.

GROS' method (see ROMEIS, "Taschenbuch der mikroskopischen Technik," 12 auf. 1928; R. OLDERBOURG, Munchen u. Berlin) is valuable, as by it staining of the connective tissue fibres can be avoided. Denny-Brown, who has modified this method for use on celloidin material, recommends the following technique: Celloidin sections up to 50 μ in thickness are placed in distilled water for half to one hour to remove all traces of alcohol. They are then transferred to 20 per cent. silver nitrate for one hour in the dark. Frozen sections are first treated with pure pyridine or with alcohol for twenty-four hours and then washed for two to three hours in distilled water until there is no odour of pyridine in order to prevent staining of the myelin sheaths. In celloidin imbedding passage though absolute alcohol suffices to overcome this disadvantage, but if desired the material may be treated with pyridine before imbedding. Sections are transferred from the silver bath, without washing, to a 20 per cent. solution of formalin neutralised with magnesium carbonate and are left there until no further white cloud appears, the solution being renewed two or three times. The reducing solution is prepared by adding strong ammonia drop by drop to 5 to 15 c.c. of 20 per cent. solution of silver nitrate until the precipitate which forms just disappears and then adding 1 small drop of ammonia for each cubic centimetre of silver taken. The sections are washed quickly in distilled water before this bath, but if the material proves difficult to stain, try transferring a section or two without washing. The reduction must be controlled under the low power of the microscope, the reducing bath being poured into a small Petri dish or watch-glass for the purpose. If nuclei and connective tissue begin to stain first, or before the axis cylinders are fully stained, add a drop of ammonia to the reducing bath. If no axis cylinders stain after ten minutes, add one drop of 20 per cent. formol to the bath. Sections must be kept fully immersed as evaporation of ammonia may cause overstaining of any part of the section which comes to the surface. After staining, the sections are placed in a 20 per cent. solution of ammonia for at least a minute (five minutes for thick sections). They are then transferred to 1 per cent. acetic acid for the same length of time and subsequently toned in 0·2 per cent. gold chloride, fixed in hyposulphite and washed in distilled water. They may be counterstained with van Gieson for a few seconds or with ½ per cent. thionin or toluidin for a minute. They may also be counterstained with both iron hæmatoxylin and van Gieson if desired.

DAVENPORT (*Arch. Neurol. and Psychiat.*, xxiv, 1930, p. 690) coats celloidin sections from spirit with 2 per cent. celloidin after mounting them on albuminised slides and then puts them into 80 per cent. alcohol for a few minutes before placing the slides in a silver bath prepared by dissolving 10 grm. of silver nitrate in 10 c.c. of distilled water, adding 90 c c. of 95 per cent. alcohol and 5 to 7 drops of approximately normal nitric acid to each 50 c.c. of silver solution. The slides are left in the silver bath until the sections are light yellow in colour (usually over-

night). They are rinsed quickly in absolute alcohol and reduced in pyrogallic acid, 5 grm.; neutral formalin, 5 c.c.; 95 per cent. alcohol, 100 c.c.; 50 per cent. commercial dextrin, 5 drops. Development usually takes about two minutes, and if the developer be kept turbid by the addition of a few drops of the dextrin from time to time many sections can be reduced without renewal of the developer. The slides are then passed through two or three changes of 95 per cent. alcohol, absolute alcohol and ether, xylol and mounted in balsam. Clearing of the background may be effected with acid sodium hyposulphite, and if desired, sections may be toned with gold chloride.

Kernohan (*Trans. Am. Micros. Soc.*, xlix, 1930, p. 58) cuts frozen, paraffin or celloidin sections at 6–15 μ, and after washing thoroughly in distilled water immerses them for an hour in 20 per cent. silver nitrate. The sections are washed quickly twice and transferred to a solution prepared by adding ammonia drop by drop to 10 c.c. of 20 per cent. silver nitrate until the precipitate is almost dissolved. Excess of ammonia is to be avoided and the solution is filtered before use. Sections stay in the silver bath from one to four minutes and are washed rapidly before reduction in 10 per cent. neutral formalin. It is often advisable to remove celloidin from celloidin imbedded material before impregnating with silver.

Trelles (*Rev. Neurol.*, i, 1932, p. 459) uses a similar method with celloidin sections; and Reumont (*Rev. Neurol.*, ii, 1931, p. 53) stains frozen sections similarly after a bath of nicotinised alcohol.

See also Schultze and Stohr (*Anat. Anzeiger*, liv, 1921, p. 529) and Loughlin (*Arch. Neurol. and Psychiat.*, xxxiii, 1935, p. 616).

The *methylen blue intra-vitam method* is important, and may be usefully employed for the study of neurofibrils. See the processes of Apáthy, Dogiel, and Bethe in Chapter XVI.

CHAPTER XLI *

AXIS-CYLINDER AND DENDRITE STAINS (GOLGI AND OTHERS)

1025. Introduction. There are three chief methods for the anatomical (§ 977) study of axis-cylinders and nerve-cell processes, viz., the methylen blue intra-vitam method, the bichromate and nitrate of silver, and the bichromate and sublimate methods of GOLGI. The methylen blue method has already been described in Chapter XVIII. (§§ 376 *et seq.*), and only a few points remain to be dealt with here. These, together with some other methods suitable for similar purposes, will be given at the end of this chapter, the principal object of which is the description of the GOLGI methods.

1026. The Methods of GOLGI. There are two methods of Golgi, viz., the **Bichromate and Nitrate of Silver Method** and the **Corrosive Sublimate Method.**

The bichromate and nitrate of silver method has been worked out by GOLGI in *three* forms—the *slow* process, the *rapid* process, and the *mixed* process.

The rapid process is the one mostly used at the present time, and it may be regarded as the classical method of inquiry into the general morphology and distribution of nerve-cells and their processes in hardened tissues. One must, however, remember that extremely delicate results may be obtained by both the mixed process and the corrosive sublimate method, and that use should be made of them also, particularly for the study of the finer relations of the nervous elements.

General Characters of the Impregnation. The preparations have not in the least the appearance of the usual stains, and are even very different in aspect from those obtained by the ordinary methods of impregnating with silver or gold. The impregnation is a partial one, by which is meant that of all the elements, whether nervous or not, that are present in a preparation, only some are coloured. This is one of the great advantages of the method, for, if all the elements present were coloured equally, one would hardly be able to follow any one of them for more than a very short distance. Golgi's method selects from among the elements present a small number which it stains with great intensity and very completely; that is to say, they are very clearly separated

* Revised and in great part re-written by C. D. F.

throughout a great distance from those elements which have remained uncoloured.

Axis-cylinders are *generally* impregnated only as long as they are non-medullated. In the adult the method stains nerve-cells and their processes so far as these are not myelinated; but if it be wished to impregnate throughout a great length the axis-cylinders, their arborisations and collaterals, the method is best applied to embryos or new-born animals, at a time when nerve-fibres have not yet become surrounded by their myelin sheath.

Nervous tissue is not the only thing that is impregnated in these preparations: neuroglia, connective tissue, fibrils, etc., also become stained, and the method has been applied with success to the study of bile capillaries, gland ducts, and the like. Both on account of this peculiarity and of the fact that the impregnation may be limited sometimes to certain elements, sometimes to others, care should be exercised in the interpretation of the results obtained. A further source of possible error is found in the formation of precipitates which may, up to a point, simulate dendrites and other structures.

The Golgi methods have been applied with success, also, to tissues of invertebrates—insects, *Lumbricus*, *Tubifex*, *Helix*, *Limax*, *Eistomum*, *Astacus*, *Actinida*, etc.

The methods have been described at length by GOLGI in *Riv. Sperim. Freniatr.* I, 1875; *Arch. p. le Sc. Med.*, iii, 1878; *Arch. Ital. Biol.*, vii, 1886, pp. 15 *et seq.*; *Opera Omnia*, Milano, I and II, 1903, and many other publications. A valuable account of the rapid process has been given by v. LENHOSSÉK in his *Feinere Bau d. Nervensystems*, 2nd ed., 1895, and of both Golgi's methods and their modifications by Kallius in the art. "*Golgische Methode*," in the *Enzyk. d. mik. Technik I*, 1910.

1027. GOLGI'S **Bichromate and Nitrate of Silver Method.** SLOW PROCESS. (*a*) *The Hardening.* The tissues must be hardened in a bichromate solution. Either pure potassium bichromate may be employed or Müller's fluid. (The reaction can be obtained with Erlicki's fluid, but this is not to be recommended.) The normal practice is to use potassium bichromate, beginning with a strength of 2 per cent. and changing this frequently for fresh solutions of gradually increasing strength—2½, 3, 4, and 5 per cent. The tissue should be *as fresh as possible*, though satisfactory results may sometimes be obtained from human material collected at the P.M. table even twenty-four to forty-eight hours after death. *It should be divided into pieces of not more than* 1 *cm. or* 1½ *cm. in size.*

The most difficult point of the method consists in *finding out the exact degree of hardening*, after which the material can be successfully submitted to the further treatment. In summer good results may be obtained after fifteen to twenty days of hardening, and the material may continue to be in a state suit-

able for the silver impregnation up to thirty, forty or fifty days. In cold weather good results can seldom be obtained under a month; when this is the case, the material may continue to give good impregnations for two, three or even four months. The only way to make sure is to pass, at intervals, trial portions of the tissue into the silver nitrate solution—in summer frequently, in winter every eight or ten days—and observe whether and when the reaction has been obtained.

It is a good practice to inject the organs (see § 978) with the hardening fluid, generally 2·5 per cent. potassium bichromate, to which, according to Golgi, 5 to 6 per cent. of gelatin may be added, in which case, however, the fluid must be injected after warming it to body temperature. Stoving at a temperature of 20° to 25° C. is useful for abridging the hardening, but there is a risk of over-hardening; and Golgi thinks that the results are never quite so delicate as after hardening at room temperature.

(*b*) *Impregnation.* As soon as the pieces of tissue have attained the proper degree of hardening, they are brought into a *large quantity* of silver nitrate solution, the usual strength of which is 0·75 per cent., but 0·50 per cent. may be used for material which has not been quite enough hardened, and 1 per cent. for material that has been slightly over-hardened.

The moment the pieces are put into the silver bath an abundant precipitate is formed. This, of course, weakens the bath *pro tanto.* It is, therefore, advisable first to wash them well in a weaker silver solution until, on being put into a fresh quantity of it, no further precipitate is formed. Used solutions will do for this purpose. The final silver bath needs, generally, no further attention; but it should be changed for a fresh one if it becomes yellowish, as it sometimes does, particularly in the case of tissues which have taken up a great deal of bichromate.

It is not necessary to keep the material in the dark during the impregnation; in winter it is well to keep it in a warmed room. The time generally necessary for the impregnation is from twenty-four to forty-eight hours; but tissues may remain in the bath without hurt for days, weeks or months.

(*c*) *Preservation.* As soon as a trial has shown that a sufficiently satisfactory impregnation has been obtained, the pieces are brought into 80 to 90 per cent. alcohol. The alcohol is changed two, three or more times, until it remains transparent, even after specimens have been two or three days in it; for, in view of good preservation, it is necessary that the excess of silver nitrate should be washed out from them thoroughly.

Sections are now made (see § 1039). These are to be washed thoroughly in three or four changes of absolute alcohol and cleared, first in creosote, in which they should remain only a few minutes, then in oil of turpentine, in which they are usually left

for three to fifteen minutes, though they may be kept in it even for some days without being spoiled. They are then mounted in thick xylol-damar (rather than in balsam), *without coverslip.* Preparations mounted with coverslips in the usual way always go bad sooner or later, whilst those mounted without a cover keep well for years, especially if they are protected from dust and light.

Instead of creosote and oil of turpentine, fluid cedar-wood oil is now used in Golgi's laboratory for clearing the sections, which are then mounted, without cover, in thick cedar-wood oil. But care must be taken to leave the sections in fluid cedar-wood oil no longer than one hour or so, as otherwise they become brittle and difficult to mount. To make sure of complete dehydration and that no curling of the sections should take place in the fluid cedar-wood oil, they are quickly passed through liquid absolute guaiacol, the whole procedure being carried out as follows: A small quantity of absolute guaiacol is poured in a watch-glass and some fluid cedar-wood oil in two other small glass dishes. Two or three sections are carried from the absolute alcohol into the guaiacol by means of a perforated spatula, which is to be used for all the other passages, and cleaned at every passage. After a few seconds the sections are transferred into the first dish of fluid cedar-wood oil and there left for the time necessary to pass another two or three sections from the absolute alcohol into the guaiacol. The first batch of sections is now transferred into the second dish of cedar-wood oil, the second batch into the first cedar-wood oil and a fresh batch into guaiacol, and so on until all sections are collected in the second dish of cedar-wood oil.

For mounting the sections are lifted, one by one, by means of the same small spatula, and arranged in the order and number one may wish, either on ordinary slides, or on coverslips if the Golgi hollowed-out wooden slides are preferred for definite preservation. The excess of cedar-wood oil carried with the spatula is removed by covering the sections, after having definitely arranged them on the slides, first with a sheath of cigarette paper and then with a folded piece of filter paper, to be held by the left hand while the right is passed over it so as to press down the sections and absorb the oil. The whole manœuvre may be repeated a second time, and then a drop of thick cedar-wood oil put on each section. On the next day the oil which may have run from the sections is cleaned from the edges of the slides and a fresh drop of the thick cedar-wood oil put on the sections, to be protected from dust and light at least until the oil has become quite dry.

Preparations mounted in this way last for years unaltered; in fact, I have some which were made in Golgi's laboratory over fifteen years ago and I find that they have kept without change.

I have no experience of the use of creosote or of the mixture, originally proposed by Andriezen, of equal parts of pyridine and xylol instead of the guaiacol, but they should equally well serve the purpose.

As a general rule one makes sections of 20 to 40 μ; thicker sections of 50 to 60 μ, or more, show more than thin ones but do not seem to keep so well.

The order in which the elements of nervous tissues impregnate is generally—first, axis-cylinders, then nerve-cells, and lastly, neuroglia cells.

1028. GOLGI's **Bichromate and Nitrate of Silver Method.** RAPID PROCESS. Small pieces of very fresh tissues are hardened in a mixture of 2 to 2·5 per cent. potassium bichromate 8 parts, and 1 per cent. osmic acid 2 parts. Or, if a very quick hardening is desirable, 2 parts of 3 per cent. bichromate to 1 of 1 per cent. osmic acid. In Golgi's laboratory mixtures of 3 parts of 3 per cent. bichromate and 1 of 1 per cent. osmic acid are generally used. The tissues begin to be in a state suitable for the silver impregnation from the second or third day; in the next following days they are in a still more favourable state, but this soon declines, and is generally quite lost by the tenth or twelfth day.

The silver impregnation is conducted exactly in the same way as in the slow process, and sections are prepared and mounted in the same manner, but they should not be left in alcohol for more than an hour or so before mounting.

There is this difference, that the impregnated material cannot be preserved for any length of time in alcohol and must not remain in it for more than one or two days. But it may be kept in the silver solution until wanted for sectioning. According to v. GEHUCHTEN (*La Cellule*, vi, 1890, p. 405) pieces may be kept with advantage for many days, weeks and months in the silver nitrate solution. An abundant impregnation was found by him after many days up to six months where almost none had been seen after twenty-four to forty-eight hours only. But the material must be kept in the dark.

As to the proper duration of the hardening process in different cases, it must be pointed out that definite rules can hardly be given, while investigators can easily find out the right moment for successfully transferring the pieces into the silver bath by means of attempts made in accordance with the purpose in view and the quality of the material with which they are working. However, the following points should be borne in mind :—

Spinal cord of chick from the sixth to the tenth day of incubation—twelve to forty-eight hours in the hardening mixture (up to the fifth day the embryos may be treated whole, later the vertebral column should be dissected out and cut into two or three segments; it need not be opened). The *spinal column of new-born rats and mice* should be treated in the same way, and remain in the mixture for twenty-four hours for

spinal ganglia, or for two to six days for the cord itself. The *encephalon* of these subjects may be treated in just the same way, without being dissected out.

v. Lenhossék (*op. cit.*) recommends for *human fœtal cord* two to three days for neuroglia, three to five for nerve-cells, and five to seven for nerve-fibres and collaterals.

Cerebellum of new-born subjects three to five days in the hardening mixture.

Cerebral cortex of young subjects two to three days (mice), or as long as five (rabbit, cat); cortex of adults, eight to fifteen days. The most favourable region of the brain is the Ammon's horn, especially in the rabbit.

Retina—twenty-four to forty-eight hours in the mixture, then "double" impregnation (§ 1033).

Sympathetic. Sala, L. (*Mon. Zool. Ital.*, iii, 1892) found the inferior cervical ganglion particularly suitable for staining by Golgi's rapid process. He proceeds thus: osmium bichromate mixture, three days; quick wash in distilled water; silver bath, two to three days; further wash in distilled water and passage into the same osmium-bichromate mixture for about four days; a third impregnation can be resorted to, in which case pieces should remain in the hardening fluid for five to seven days.

Spinal cord of *larvæ of Amphibia*. The entire larvæ (best 2 to 2·5 cm. long) should be put for two to five days into the hardening mixture, and for one to two into silver nitrate.

Epidermis of *Lumbricus*. Three to six days in the mixture, and two in the silver, or double impregnation if necessary.

Nervous system of *Helix* (glia-cells). The above mixture for eight to ten days, then silver of 0·75 to 1 per cent.

As a general rule, the younger the subject the shorter should be the hardening. If it has been too short, sections will have a brownish-red opaque aspect, with precipitates, and irregular impregnation of cells and fibres. If it has been too long, the ground will be yellow, without precipitates, but with no impregnated elements, or hardly any.

This process has the advantage of great rapidity, and of sureness and delicacy of results, and it is the one that has found most favour with other workers. But for the methodical study of any given part of the nervous system Golgi himself prefers the following:—

1029. Golgi's **Bichromate and Nitrate of Silver Method.** Mixed Process. Fresh pieces of tissues are put for periods varying from two to twenty-five or thirty days into the usual bichromate solution (§ 1027). Every two or three or four days some of them are passed into the osmio-bichromate mixture of the rapid process, hardened therein for from three or four to eight or ten days, and finally impregnated with silver nitrate and subsequently treated exactly as by the rapid process.

The reasons for which Golgi prefers this process are: The certainty of obtaining samples of the reaction in many stages of intensity, if a sufficient number of pieces of tissues have been used for the purpose. The advantage of having at one's disposal a considerable time—some twenty-five days—during which the tissues are in a suitable state for taking the silver. The possibility

of greatly hastening the process whenever desired by simply bringing all the pieces over at once into the osmic mixture. Lastly, a still greater delicacy of results, particularly noticeable in the staining of axons and their collaterals.

1030. GOLGI'S **Methods for demonstrating Funnels and Spiral Filaments.** GOLGI (see REZZONICO, *Arch. p. l. Sc. Med.*, iv, 1880, p. 78; GOLGI, *Opera Omnia*, I, p. 163) puts small pieces of *spinal cord* in 2 per cent. potassium bichromate for eight to fifteen days in summer, or a month in winter. After a quick wash he transfers them into 0·50 to 0·75 per cent. silver nitrate for two or three days in summer, or eight, ten or more in winter. The pieces are then washed in 95 per cent. alcohol, dehydrated in absolute alcohol, cleared in oil of turpentine and teased therein. The preparations, mounted in dammar, must be exposed to sunlight for eight to ten days; or to diffused daylight for twenty to forty days.

For Peripheral Nerve-fibres, GOLGI (*Op. Omnia*, I, p. 162) has proposed two methods. Of these the first is a modification of his rapid process (see § 1028), and should be carried out as follows:—Tracts of peripheral nerves are cut with care not to stretch them, and put in a mixture of 10 parts of 2 per cent. potassium bichromate and 2 of 1 per cent. osmic acid. After about one hour the tract or tracts of nerves are sufficiently hardened to be further recut in pieces of about ½ cm. in length, which are put back in the same mixture. After another three hours, and successively at intervals of three hours during twenty-four hours, pieces are transferred into 0·5 per cent. silver nitrate where they may remain for any time, but no less than eight hours. Preparations are made and mounted as above.

The other method is a modification of that used for central nerve-fibres, the only difference consisting in keeping the pieces in the bichromate for a much shorter period, *i.e.* for from four hours to at most two days, and in transferring specimens into the silver bath at intervals of about three hours. After twelve to twenty-four hours preparations can be made as described above.

The preparations made by the first method show the spiral filaments very clearly, but do not keep well. The preparations made by the second method do not show the spiral filaments so completely, but are more useful for the demonstration of the funnels and last longer.

CATTANI (*Arch. Ital. Biol.*, vii, 1886, p. 345) either fixes in Flemming's fluid and teases and mounts in glycerin, or puts pieces into Golgi's bichromate and osmic acid mixture, dehydrates and passes into oil of turpentine to be changed until it remains colourless. The turpentine dissolves the myelin and leaves funnels and spiral filaments visible. Cattani also has a modified Golgi method, now superseded.

SALA (*Verh. Anat. Ges. Anat. Anz.*, 1900, p. 176) employs the Golgi-Veratti method for the intracellular network (see § 722).

See also concerning these methods, MONDINO, *Arch. p. l. Sc. Med.*, viii, p. 45.

GALLI (*Ztschr. wiss. Mikr.*, iii, 1886, p. 467) hardens peripheral nerves for eighteen to twenty days in Müller's fluid, cuts out pieces 5 to 6 mm. long, and keeps these in Müller's fluid diluted with 2 parts of water for another two days; then in glycerin acidified with acetic acid (1 drop to 1 c.c.). From this, without washing, the pieces are transferred to a watery solution of China blue, in which they are kept for fifteen to twenty minutes, according to the amount of acetic acid added to the glycerin. They are then brought into alcohol, in which teasing is begun, and then through absolute alcohol into turpentine, in which the teasing is completed. Mount in dammar.

RAMÓN Y CAJAL has successfully employed some modifications of his reduced silver and uranium nitrate methods, for which see *Trab. Lab. Invest. Biol.*, Madrid, x, 1912, p. 221.

1031. Theory of Impregnation. It was once held that the reaction depends on the formation in the tissues of a precipitate of some salt of silver. And Kallius has put forward the suggestion that this precipitate may consist of a protein-silver-chromate combination. But this seems to B. Lee incorrect (see 1913 ed.). In agreement with v. Lenhossék, he finds that the coloration is not due to a visible precipitate, but is a true stain accompanied, particularly in unsuccessful impregnations, by precipitates which not only do not help the stain, but are injurious to it. It has been maintained that the stain is merely superficial, and the method has been called an "incrustation method." But it is easy to realise that it generally extends throughout the whole thickness of the impregnated elements, though in special cases or by slight modifications of the original method, the stain may be limited to certain constituents of the nerve-cell body, such as Golgi's pericellular investment and intracellular network.

The chemical nature of the stain has not as yet been discovered.

A critical review of the Golgi method by WEIGERT may be found in *Ergebn. d. Anat.*, v, 1895, p. 7. See also HILL (*Brain*, xix, 1896, p. 1), and KALLIUS (*op. cit.*).

MODIFICATIONS OF GOLGI'S BICHROMATE AND SILVER NITRATE METHOD CONCERNING THE IMPREGNATION OF TISSUES.

1032. Instead of potassium bichromate, ammonium bichromate has been recommended by GOLGI and sodium bichromate by KALLIUS. Both these salts appear to penetrate more quickly

into the tissues than potassium bichromate. According to STRONG (*N. Y. Acad. Sc. Proc.*, xiii, 1894) lithium bichromate hardens more rapidly than potassium bichromate. The influence on the reaction of the bichromates of ammonium, sodium, calcium, magnesium, rubidium, lithium, zinc, and copper, has been investigated by L. SALA (see KALLIUS, *op. cit.*, i, p. 564), but he came to the conclusion that they do not offer any particular advantage, with the exception of calcium bichromate, this last to be preferred for the staining of the tangential fibres of the cerebral cortex.

RAMÓN Y CAJAL (*Ztschr. wiss. Mikr.*, vii, 1890, p. 332) gives 3 per cent. as the strength of the bichromate in the mixture for the rapid process, but in numerous other places has given it as 3·5 per cent. The latter strength has been adopted by many workers for the rapid process, and the mixture containing this proportion of bichromate is generally known as the RAMÓN Y CAJAL mixture.

1033. RAMÓN Y CAJAL'S **Double-Impregnation Process** (*La Cellule*, viii, 1891, p. 130). Sometimes the usual rapid method fails to give a good impregnation. This, however, may frequently be obtained by putting the tissues back for a day or two into the osmium-bichromate mixture used for the first hardening, or into a fresh but weaker one containing 2 parts of 1 per cent. osmic acid and 20 parts of 3 per cent. potassium bichromate. Tissues are then washed quickly with distilled water or with a weak solution of silver nitrate, and put for a second time into the silver bath, where they should remain from thirty-six to forty-eight hours. It is important to find out the proper duration of the first hardening. If it has been too long (four days) or too short (one day) the second impregnation will not succeed. In this case a third impregnation may be resorted to, the objects being again treated with the weak osmium-bichromate mixture and then again with the silver nitrate solution. I find that this modification, which is the most important that has hitherto been made, gives excellent results if one proceeds by tests, viz., re-transferring into the weak osmium-bichromate mixture those pieces in which the reaction has been found to have succeeded to some extent.

1034. KOLOSSOW'S **Modification** (see ZUSCHTSCHENCO, *Arch. Mikr. Anat.*, xlix, 1897). Tissues are hardened for one to seven days in 3 to 5 per cent. potassium bichromate containing 0·25 per cent. of osmic acid. They are then washed quickly in distilled water, dried with filter paper and transferred for two to three days into a bath of 2 to 3 per cent. silver nitrate to which 0·25 to 0·5 per cent. of osmic acid has been added. This is a good modification for sympathetic ganglia.

1035. GOLGI'S **Processes for the Rejuvenation of Over-hardened Tissues.** Tissues which have been too long in the osmium-bichromate mixture will no longer take on the silver impregnation.

They can, however, be made to impregnate by one or other of Golgi's so-called processes of rejuvenation. These can be carried out in various ways given here with sufficient detail, *as they may be of great use* not only for rejuvenating ordinary pieces of central nervous system, but also, and particularly, for the staining of nerve-endings in glandular and other tissues, internal apparatus, spiral filaments of peripheral nerve-fibres, etc.

Golgi at first suggested washing the over-hardened pieces in a half-saturated solution of copper acetate until they no longer give a precipitate, afterwards putting them back again for five or six days into the osmium-bichromate mixture, and subsequently transferring them into the silver nitrate solution.

Later he advised leaving tissues in 3 to 4 per cent. copper sulphate or 1 to 2 per cent. arsenic acid. After one, two and three days some pieces are brought back into the osmium-bichromate mixture in which they have been hardened, or into a weaker one, proceeding further as in the rapid process, viz., as if the pieces had been freshly fixed in the osmium-bichromate mixture.

More recently Golgi appears to have preferred mixtures of equal parts of 2 or 3 or 4 per cent. copper sulphate or acetate and 4 to 5 per cent. potassium bichromate, filtering them if copper acetate was used, and treating the pieces as stated above. As a rule these copper acetate and potassium bichromate mixtures ought to be tried first and in preference to others. As with other points of Golgi's methods, so also in this case, one must proceed by tentative experiments, according to the purpose of one's investigation and the quality of the material in hand, but chiefly according to the length of time during which the tissues have been left in the osmio-bichromate solution.

See on this subject Sacerdotti, *Intern. Monatschr. Anat.*, xi, 1894, p. 326 ; Golgi, *Cinquant. Soc. Biol.*, 1899, p. 514, and *Opera Omnia*, II, 1903, p. 677 ; Fusari, *Tratt. Elem. Istol. Tecn. Istol.*, Torino, 1909 ; Sala, G., *Anat. Anz.*, xviii, 1900, p. 176 ; Gemmelli, *Anat. Anz.*, 1913, p. 444.

1036. Formaldehyde Modifications *of* Golgi's *Bichromate and Nitrate of Silver Method.* Many investigators have found that formaldehyde can take the place of the osmic acid in the osmio-bichromate mixture of the rapid process. This has certain advantages : A cheap reagent is employed instead of the expensive osmic acid. Pieces much larger than by Golgi's original process may be used. The stage of hardening favourable for a good impregnation lasts longer, *i.e.*, formalin-bichromate mixtures do not over-harden. Moreover, the formaldehyde modifications can be usefully resorted to for impregnating nervous tissues of adult or young subjects, as well as for material which after repeated attempts has been found impervious to the osmic mixtures. How-

ever, it should be remembered that many investigators have failed to obtain good results by the formaldehyde methods and that they are unsuitable for embryonic specimens.

Hoyer, Jun. (*Anat. Anz.*, ix, 1894, p. 236) was the first to point out that material fixed in formalin could be used for carrying out Golgi's method.

Lachi (*Monit. Zool. Ital.*, v, 1895, p. 15) used, at first, to harden tissues for five to nine days in equal parts of 20 per cent. formalin and 6 per cent. potassium bichromate. Afterwards (*Anat. Anz.*, x, 1895, p. 790) he adopted the mixture proposed by his pupil Dell'Isola (*Boll. Acc. Med. Genova,* 1895, No. 2) of equal parts of 10 per cent. formalin and 10 per cent. potassium bichromate, with the addition of 1 part of 1 per cent. osmic acid to every 10 of the mixture, this last formula being particularly suitable for quick work, as forty-eight hours afterwards pieces can be already transferred into the silver bath.

Strong (*Anat. Anz.*, x, 1895, p. 494) suggested fixing pieces of brain of adult specimens in mixtures of 100 volumes of 3·5 per cent. potassium bichromate and from 2½ to 5 volumes of formalin. One or more pieces are, during several days, daily transferred into 1 per cent. silver nitrate. Or the tissues are left for one to two days in the above formalin-bichromate mixture and then passed into a fresh one consisting of 2 volumes of 5 per cent. potassium bichromate and 1 volume of formalin; after another twelve to twenty-four hours all the pieces are transferred into the silver bath.

Durig (*ibid.*, p. 659) obtained good results by fixing ½ cm. thick pieces in 3 per cent. bichromate containing 4 to 6 per cent. of formalin, and hardening therein for three days. After silvering for two days, the pieces are brought back into the fixing mixture and one proceeds as in Ramón y Cajal's double impregnation process.

Fish (*Proc. Amer. Micr. Soc.*, xvii, 1895, p. 319) uses 2 c.c. of formalin for every 100 c.c.of 3 per cent. potassium bichromate, and leaves tissues three days in this fluid, and another three days in 0·75 per cent. silver nitrate; or, with advantage, Müller's fluid, 100 c.c.; 10 per cent. formalin, 2 c.c.; 1 per cent. osmic acid, 1 c.c.; silvering as above.

Kopsch (*Anat. Anz.*, xi, 1896, p. 727) uses 4 parts of 3·5 per cent. potassium bichromate and 1 of formalin; after twenty-four hours he transfers all pieces to pure 3·5 per cent. bichromate for at least two days (retina) or three to six (central organs). He finds that by this means, precipitates are almost entirely avoided. B. Lee (1913 ed.) confirms this, but points out that the method gives a too abundant impregnation of capillaries.

Gerota (*Intern. Monatsch. Anat.*, xiii, 1896, p. 108) first hardens brains for a week or two in 5 to 10 per cent. formalin,

then puts small pieces for three to five days into 4 per cent. bichromate, and lastly transfers these into the silver bath, where they are left with advantage for ten to twenty days.

Bolton (*Lancet*, 1898 (1), p. 218; *Journ. R. Micr. Soc.*, 1898, p. 244) has obtained good results from brains of cats and half-grown kittens placed whole in 5 per cent. formalin and from human brains hardened whole in formalin of the same strength for two to twelve months. Small pieces are then cut out, and placed into 1 per cent. ammonium bichromate and left therein for from a few hours up to five days, some being transferred at intervals into 1 per cent. silver nitrate.

Schreiber (*Anat. Anz.*, xiv, 1898, p. 275) obtained good results from appendages of Crustacea impervious to the osmic mixture, with 5 parts of 2·5 per cent. potassium bichromate to 1 of 4 per cent. formalin, or 1 part of 2·5 per cent. bichromate to 2 of 5 per cent. formalin, the specimens remaining for one day in the first mixture and for two in the second.

Similarly Dubosq (*Arch. de Zool. exp.*, vi, 1898–90).

Smirnow (*Arch. mikr. Anat.*, lii, 1898, p. 201) fixes the cerebellum of a freshly-killed animal in 5 per cent. potassium bichromate 4 parts, and formalin 1 part, for one to eight weeks. He then divides the organ in two halves and places them into pure 3·5 per cent. bichromate, where they are left for another two to five weeks. Of the two halves one may be used for Weigert's myelin stain; the other is divided in pieces 1 to 2 cm. thick, and these put for one to one and a half weeks into a mixture of 5 per cent. potassium bichromate, 5 parts, and 2 per cent. osmic acid, 1 part. Pieces are then transferred, at first into a weak silver bath and then into the usual 1 per cent. silver nitrate solution. The method gives good results also in human brains of adult subjects.

Odier (*La Rachicocainisation*, Genève, 1903, p. 27) takes 100 c.c. of Müller's fluid with 2 c.c. of undiluted formalin and 1 c.c. of 1 per cent. osmic acid. The mixture should be kept in the dark and made up at the instant of using it. Odier finds that formalin-bichromate mixtures generally afford a more abundant impregnation with fewer precipitates than the osmio-bichromic ones.

Brookover (*Journ. comp. neurol.*, xx, 1910, p. 49) finds useful for adult specimens a preliminary fixation in 4 per cent. "formaldehyde," neutralised with lithium carbonate or ammonia, before carrying out Golgi's rapid process.

1037. Other Modifications. Vassale and Donaggio (*Monit. Zool. Ital.*, vi, 1895, p. 82) harden pieces of at most 1 cm. in thickness for fifteen to twenty days in a mixture of 5 parts of acetic aldehyde and 100 of 3 to 4 per cent. potassium bichromate, changing the fluid as soon as it has become dark. The rest as Golgi.

Ramón y Cajal (*Rev. Trim. Histol.*, No. 2, 1888, note) found that the addition of a very little formic acid to the silver bath facilitated reduction. According to van Gehuchten (*La Cellule*, vii, 1891, p. 83) 1 drop of the acid should be added to every 100 c.c. of the silver nitrate solution. But the practice is now generally abandoned.

Martinotti (*Rif. med.*, 1887; *Ztschr. wiss. Mikr.*, v, 1888, p. 88) pointed out that Golgi's method can be successfully carried out on relatively large pieces by using unusually large quantities of silver nitrate solution with 5 per cent. glycerin added to it, and by keeping this for thirty days at a temperature of 25° C. to impregnate nerve-cells, and of 35° to 40° C. to stain the neuroglia.

Andriezen (*Brit. Med. Journ.*, i, 1894, p. 909) found useful for human brain to suspend thin slices of 2 to 4 mm. in diameter in 95 c.c. of 2 per cent. potassium bichromate to which after ten to fifteen minutes 5 c.c. of 1 per cent. osmic acid are added. The mixture is kept in the dark and after twenty-four hours changed for a fresh one made up with 90 c.c. of $2\frac{1}{2}$ per cent. bichromate and 10 c.c. of 1 per cent. osmic acid. After another two days the mixture is changed over again for one made according to the proportions given by Golgi (3 per cent. potassium bichromate, 80 c.c.; 1 per cent. osmic acid, 20 c.c.). Pieces are transferred into the silver bath after three and a half days (for nerve-cells and neuroglia) up to six days. They are washed for five to fifteen minutes in $\frac{3}{4}$ per cent. silver nitrate, and then put into a solution of silver nitrate of the same strength, but to which 1 drop of formic acid to every 100 or 120 c.c. of solution has been added. The whole is kept in an incubator at 25° to 27° C. for about three days, changing the silver bath after the first twenty-four hours. The same author advised, for the impregnation of neuroglia (*Intern. Monatschr. Anat.*, x, 1893, p. 533), adding 1 drop of a saturated solution of chromic acid and 1 drop of formic acid to the first hardening bath.

Berkeley (*Johns Hopkins Hosp. Rep.*, vi, 1897, p. 1) hardens tissues in Müller's fluid until they are of sufficient consistency to admit of fairly thin sections (about two weeks at room temperature). The portions of the brain selected are cut into slices 3 mm. thick and immersed for about three days in a mixture of 3 per cent. potassium bichromate, 100 parts, and 1 per cent. osmic acid 30 parts. For the impregnation, tissues are removed from the hardening fluid, dried a little with filter paper, washed in a weak solution of silver nitrate, and put for no less than two to three days into a freshly-prepared solution of 2 drops of 10 per cent. phosphomolybdic acid and 60 c.c. of 1 per cent. silver nitrate, which in winter should be kept at a temperature of about 26° C.

Hill (*op. cit.* § 1031) uses, instead of silver nitrate, a $\frac{3}{4}$ per cent. solution of silver *nitrite*, with 0·1 per cent. formic acid added.

Gudden (*Neurol. Centrbl.*, xx, 1901, p. 151) uses the lactate of silver (sold as "actol"), and finds it more penetrating.

1038. Avoidance of Precipitates. Golgi's method frequently gives rise to the formation at the surface of the pieces of irregular and sometimes voluminous precipitates, which destroy the clearness of preparations. To minimise this, Sehrwald (*Ztschr. wiss. Mikr.*, vi, 1889, p. 456) pours 10 per cent. gelatin, which is just liquid, into a paper box, imbeds the tissues in it with the aid of a little heat, and brings them therein into the silver bath; or the tissues are coated with gelatin by dipping and cooling several times. After the impregnation is completed the gelatin

is removed, before cutting, by means of warm water saturated with silver chromate. Mann (*Physiol. Histol.*, 1902, p. 276) finds that the method gives good results provided the gelatin is not rendered insoluble by the action of light. To prevent this he proceeds thus: Either in the photographic dark room or in the evening, by artificial light, tissues, tied loosely to a thread, are immersed three times into liquefied 10 per cent. gelatin, and, as soon as this has set, they are put into the silver bath, keeping the latter in some dark place. It appears that surrounding a tissue with gelatin makes the impregnation slower, and for this reason Mann allows a day longer for the silver bath.

Martinotti (*op. cit.*) covers pieces with a layer of a pap of filter paper and distilled water.

Athias wraps tissues in wafer papers.

Ramón y Cajal covers them with a layer of congealed blood, which need not be removed before cutting, or with celloidin or peritoneal membrane. See " Retina."

MODIFICATIONS CONCERNING THE PRESERVATION OF THE PREPARATIONS

1039. Cutting. As pointed out in § 1026, one of the chief qualities of Golgi's method consists in allowing one to follow nerve-cell processes for a great distance. Evidently this cannot be done with very thin sections; and as sufficiently thin ones can be obtained without imbedding, the general practice is simply to wash the pieces taken from the silver bath with distilled water, fix them with gum to a cork or wooden cube, put the whole into alcohol for a little while to harden the gum, and cut by means of a sliding microtome without imbedding.

But quick imbedding, particularly in celloidin, is quite possible, and should be resorted to for material either brittle or otherwise difficult to cut. Pieces of tissue as small as possible are brought in the course of about two hours through the ascending series of alcohols into absolute alcohol; after having changed this a couple of times, pieces are transferred for another one or two hours to thin celloidin, then coated with thick celloidin, and by means of this fixed to a wooden cube, the celloidin being a little hardened by means of chloroform vapour, as usual. The whole is left for a little while in 70 per cent. alcohol, and sections made in the usual way. If these operations are started in the morning, when going into the laboratory, pieces are ready for cutting at about 2 p.m., sufficient time remaining for the further treatment of the sections according to the directions given above (§ 1027). Care should be taken, of course, not to transfer the sections into absolute alcohol if it is not considered safe to dissolve the celloidin. In this case dehydration can be carried out as usual up to 98

per cent. alcohol, and the sections transferred into fluid absolute guaiacol and cedar-wood oil as already described in § 1027.

Imbedding in paraffin is also possible, but results are usually rather poor, and one should have recourse to it only for special objects, such as muscles (see Veratti, *Mem. R. Inst. Lomb. Sc.*, xix, 1902, p. 87). In any case tissues should be passed quickly through the lower grades of alcohol, and remain only a few hours in 95 per cent. and absolute alcohols. They should be cleared with cedar-wood oil, as xylol and similar reagents may be injurious to the silver impregnation. One should transfer pieces directly into paraffin of as low a melting point as possible. According to Brookover (*op. cit.*), cedar-wood oil should be used over and over again, as it becomes saturated with silver nitrate.

1040. Mounting. As pointed out in § 1027, Golgi preparations do not keep well if mounted under a cover-glass in the usual way. How and why this happens it is very difficult to say. Though an elaborate discussion between Sehrwald (*Ztschr. wiss. Mikr.*, vi, 1889, p. 443), Samassa (*ibid.*, vii, 1890, p. 26), and Fish (*ibid.*, viii, 1891, p. 168) has furnished the net practical result that watery fluids should be avoided as much as possible during the after-treatment, it is not clear why preparations should deteriorate, when mounted under a cover-glass in thick cedar-wood oil or neutral balsam; while Mann (*op. cit.*, p. 277) states, on the other hand, that sections keep well if mounted under a cover-glass in Price's No. 1 pure neutral glycerin.

For these reasons the general practice is to mount sections without a cover, either on ordinary slides or on cover-glasses to be inverted for study over the aperture of a hollowed-out wooden slide.

If mounting under a cover is desirable, this should either be raised free of contact with the slide by means of wax feet or the like, or the balsam of the mount should be rendered perfectly anhydrous by carefully heating it on the slide with the section in it, until it immediately sets hard on cooling, when a slightly warmed cover can be applied. This last method is also recommended by Huber (*Anat. Anz.*, vii, 1892, p. 587). B. Lee (see previous editions) advises keeping the preparations uncovered until the sections have become quite dry and the balsam, applied from time to time in thin layers, quite hard, and then to cover them with a warmed cover-glass, this being slightly pressed down on the sections.

Various processes have been devised for mounting Golgi's preparations at once under a cover, but none of them give really satisfactory results. One should have recourse to them either for special objects, or if counterstaining with carmine or hæmatoxylin, or by Weigert-Pal's method, or the like, is particularly desirable. In this case one of the following methods may be employed :—

Greppin (*Arch. Anat. u. Entwick., Anat. Abth., Supp.*, 1889, p. 55) treats sections for thirty to forty seconds (until whitish) with 10 per

cent. hydrobromic acid, washes them in several changes of water, dehydrates, clears with clove oil and exposes them for ten to fifteen minutes to sunlight.

Obregia (*Virchow's Arch.*, cxxii, 1890, p. 387) transfers sections into a mixture of absolute alcohol, 10 c.c., and 1 per cent. gold chloride, 10 drops, to be previously exposed to diffuse daylight for half an hour. Sections are then passed into it and put in a dark place. After fifteen to thirty minutes they are washed successively in 50 per cent. alcohol, distilled water, 10 per cent. sodium hyposulphite (five to ten minutes), and repeatedly changed distilled water. They may be then counterstained, dehydrated and mounted in balsam under a cover.

Kallius (*Anat. Hefte.*, ii, 1893, p. 271) uses 230 c.c. of distilled water and 20 c.c. of commercial hydroquinone solution (hydroquinone 4 grm., sodium sulphite 40 grm., potassium carbonate 75 grm., distilled water 250 c.c.). The solution is further diluted before using with one-third to one-half its volume of absolute alcohol and the sections (freed from unreduced silver by washing them in many changes of alcohol) left in it for several minutes. Here they become dark-grey to black, and are then transferred for ten to fifteen minutes into 70 per cent. alcohol, for five minutes into 20 per cent. sodium hyposulphite, and for twenty-four hours into a large quantity of distilled water. Counterstain, dehydrate, clear and mount as usual.

Eberth and Runge (*Arch. mikr. Anat.*, xlvi, 1896, p. 370) have successfully used a process similar to that of Greppin. They convert the silver impregnation into silver chloride by keeping sections in chlorine water for fifteen to twenty minutes, and they then reduce the white silver chloride, either through exhibition to sunlight just before mounting, or by means of Kallius' process.

Bolton (*op. cit.*) has obtained good results with Kallius' process applied to his formol-bichromate modification.

Curreri (*Anat. Anz.*, xxxii, 1908, p. 432), after fixing by Kallius' method, tones in 0·7 grm. of gold chloride, 3 grm. of sodium acetate and 100 c.c. of water.

Zimmermann's process (*Arch. mikr. Anat.*, lii, 1898, p. 554). Paraffin sections of formol-Golgi material are brought from alcohol into a large quantity of a mixture of 1 part of physiological salt solution and 2 parts of 96 per cent. alcohol. They are kept in motion therein for ten to fifteen minutes, after which they are brought into 75 to 96 per cent. alcohol in a bright light until they have become dark (about half a day); or sections are left for half to one hour in 100 c.c. of absolute alcohol to which a few drops of ammonium hydrosulphide have been added. In the first case the silver deposit becomes converted into silver chloride, in the second into silver sulphide. Later (*Arch. mikr. Anat.*, lxxviii, 1911, p. 199) he reduces for several hours in 20 c.c. of saturated solution of sodium carbonate (made up with 50 per cent. alcohol) to which 0·5 grm. of *adurol* are added. These processes are useful for studying the inter-relationship between gland-ducts and gland-cells (stomach, liver) if the silver chloride sections are afterwards stained with thionin or toluidine blue or safranin, the sulphide sections with Delafield's hæmatoxylin, and the adurol ones with hæmalum or alum cochineal.

For toning, fixing and counterstaining sections of tissues treated by the sublimate method and the like, see next paragraph.

THE SUBLIMATE METHOD

1041. Golgi's Bichromate and Sublimate Method (*Arch. Sc. Med.*, iii, 1878; *Rend. R. Inst. Lomb. Sc.* (2), xii, 1879, p. 205;

and (2), xxiv, 1891; *Arch. Ital. Biol., op. cit.*, § 1026; *Rif. Med.*, 1891; *Opera Omnia*, I, p. 143, and II, pp. 505 and 607). For hardening, use either a solution of potassium bichromate progressively raised from 1 to 3 per cent., or Müller's fluid. It is best to take small pieces of tissue, large quantities of hardening fluid, and change the latter frequently. But the reaction can be obtained with much larger pieces, even entire hemispheres. In this case the brain should at first be treated with "*repeated*" injections of the fixing agent, or this should be injected from the carotid or the aorta. Pieces, particularly if small, begin to be ready for the subsequent treatment eight to ten days afterwards, but it is advisable to wait until the twentieth or thirtieth day of immersion, this being not injurious if prolonged for several months; it is, on the contrary, to be recommended if the pieces are uncommonly large.

When it is thought that the tissues have been hardened enough, they are passed directly from the bichromate into 0·5 to 1 per cent. mercury chloride. One generally prefers weak solutions (0·5 per cent.) if pieces have been left in the fixing fluid for a relatively short period, having recourse to the stronger ones (1 per cent.) for materials which have been hardened for many weeks or months. The sublimate solution must be changed at first every day, and later as often as it comes yellowish. At the end of the reaction pieces will be found decolourised and almost with the aspect of fresh tissue. To obtain a good reaction, about ten days of immersion in the mercury chloride are necessary if pieces are small, longer periods, and even months, being required for large pieces and entire hemispheres. Particularly fine results were obtained by Golgi from brains which had been kept in 1 per cent. sublimate for as long as two years.

The reaction may be said to have begun by the time tissues are nearly decolourised. From that time onwards sections may be made and mounted if successful.

Imbedding is not necessary, but in many cases desirable. It can be easily carried out by washing pieces in many changes of alcohol of ascending strengths and imbedding them in celloidin. Sections, however made, must be repeatedly washed with distilled water, otherwise they will soon be spoilt by the formation of opaque granules and needle-like crystals which very much hinder proper observation. After dehydrating, sections can be passed through creosote and turpentine and mounted, preferably without a cover-glass, in dammar or balsam.

It is, however, preferable to treat sections by the following fixing-and-toning process which was suggested by Golgi for transforming the whitish mercury impregnation (to which the reaction is due) into a full-black stain, much more suitable for observation under high power. Moreover, the process helps in

preventing the formation of opaque precipitates, and allows of mounting in the usual way without any danger of spoiling the specimens.

One proceeds thus: Sections of pieces imbedded in celloidin are thoroughly washed in many changes of water, and then transferred for a few minutes into a photographic fixing and toning bath to be prepared at the moment of using, as follows :—

Solution A.

Distilled water	1000 c.c.
Sodium hyposulphite	155 gr.
Potassium alum	20 ,,
Ammonium thiocyanate	10 ,,
Sodium chloride	40 ,,

Allow to stand for eight days and then filter.

Solution B.

Gold chloride	1 gr.
Distilled water	100 c.c.

For use take 50 c.c. of sol. A, 7 c.c. of sol. B, and 40 c.c. of old combined bath.

From the fixing and toning bath sections are transferred into distilled water and again thoroughly washed; they are then slightly counterstained with an acid solution of carmine diluted with some alcohol, dehydrated, cleared, and mounted in the usual way.

The elements stained by the method are: (1) Nerve-cells with all their processes and ramifications. (2) Nuclei, which is not the case with the silver process. (3) Neuroglia cells. But the reaction in this case is far less precise and complete than that obtained by the silver method. (4) Blood-vessels, and particularly their muscle fibre-cells.

The method gives particularly good results with cerebral cortex and Ammon's horn, very poor ones with the cerebellum and spinal cord. It is superior to the silver method in so far that the reaction can always be obtained with certainty in a certain time; that the preparation can be preserved by the usual methods; that large pieces of tissue can be impregnated. Moreover, it is cheaper and may give a more abundant and finer impregnation than even the rapid process.

1042. Modifications of Golgi's Bichromate and Sublimate Method. Mondino (*Ztsch. wiss. Mikr.*, ii, 1885, p. 157) has obtained good results from even whole human brain treated according to Golgi's original method.

Flatu (*Arch. mikr. Anat.*, xlv, 1895, p. 158) fixes whole human brain in 3 to 4 per cent. potassium bichromate. After two or three months slices ½ cm. thick and 1 to 2 cm. wide are brought into 0·1 per cent. mercury bichloride to be changed every two to three days for the first three weeks or so. Pieces are ripe for cutting after nine to twelve months, at which time they are washed and imbedded

in celloidin. Sections are passed through alcohols, cleared in carbol-xylol and mounted in balsam.

Pal (*Erratum* "Tal") (*Ztschr. wiss. Mikr.*, iv, 1887, p. 497) converts the whitish mercury impregnation into a black one by treating sections with 1 per cent. sodium sulphide. They may then be counterstained with Magdala red.

Golgi's sublimate method may be combined with Weigert's myelin stain (see Pal, *Wiener med. Jahrb.*, N.F. 1, 1886, p. 619, and the abstract of this paper in *Ztschr. wiss Mikr.*, iv, 1887, p. 92, in which Edinger pointed out that the mercury impregnation can be turned black by treating sections with diluted ammonia).

Flechsig (*Arch. Anat. Phys., Physiol. Abth.*, 1889, p. 537) has published a rather complicated combination of Brama's Guinea red-wood process for medullated nerve-fibres and Golgi's sublimate method, as slightly modified by Held.

1043. Cox's **Process** (*Arch. mikr. Anat.*, xxxvii, 1891, p. 16). This is the most important of all modifications of Golgi's bichromate and sublimate method. Cox found that the sublimate and bichromate can be used together, and that potassium chromate can be usefully added to the mixture in order to reduce the normally acid reaction of the bichromate, as otherwise axis-cylinders are not impregnated. He used a fluid consisting of 20 parts of 5 per cent. potassium bichromate, 20 parts of 5 per cent. corrosive sublimate, 16 parts of 5 per cent. potassium chromate, and 30 to 40 parts of distilled water. To prepare it, the bichromate and sublimate are mixed together, the chromate diluted with the water and added to the mixture.

One generally uses small pieces of tissues, but also relatively large ones can be employed, and whole brains of small animals particularly if some of the fluid has been previously injected through the carotid or aorta. The duration of the impregnation is from two to three months, but material can be left in the mixture for much longer, certainly without danger and, very likely, with advantage.

Mann (*op cit.*) recommends warming the mixture to the temperature of the incubator and diluting it to one-half the strength advocated by Cox, particularly for material of adult subjects. Portions of the brain measuring 1 cm. in thickness or entire brains of young animals are placed by him on cotton-wool in this solution and left in the incubator for twenty-four hours, when the solution is changed. After a second change on the third day the vessel (which should contain the mixture in proportion of 30 : 1 of the brain) is sealed with vaseline and left in the incubator for at least a month, but preferably for two. Da Fano finds this way of carrying out the Golgi-Cox method very good, but, after incubating for a month or so, prefers keeping the vessel at room temperature, and cutting after another two or three months or longer.

There is considerable difficulty in making and preserving

sections which ought to be made either by free hand or by means of a freezing microtome after slight preliminary washing of the pieces with water, and impregnating them with 20 per cent. dextrin for one to three days as suggested by Mann.

To convert the white mercury impregnation into a black one, Cox suggested treating the sections for an hour or two with 5 per cent. sodium carbonate, but 5 to 10 per cent. ammonia is now generally used. They are then thoroughly washed in distilled water, carefully dehydrated, cleared by one of the usual ways, and mounted, without a cover, either in thick xylol balsam or in the original medium suggested by Cox and composed of: Gum sandarac 75 grm., camphor 15, oil of turpentine 30, oil of lavender 22·5, alcohol 75, castor oil 5 to 10 drops. For examination add a drop of castor oil, and cover.

1044. Methods for rendering Golgi-Cox Preparations more permanent. Various authors (see SANDERS, 1898, *in litt. to* A. B. Lee, *Vade-Mecum*, 1913 ed., p. 433; BREMER, *Anat. Rec.*, iv, 1910, p. 263) have proposed washing tissue treated according to Cox's process in many changes of alcohol, and imbedding them in celloidin—this chiefly with the object of overcoming the difficulty of cutting brittle pieces by means of the freezing microtome, and also of rendering preparations more permanent by removing the excess of corrosive sublimate not utilised by the reaction, and which still permeates the tissues. As a matter of fact sections of pieces thus treated are very easily cut and can be transferred from one to another fluid without danger of injuring them. Moreover, they can be counterstained, and the impregnation keeps sufficiently well, particularly if sections are mounted without a cover-glass. But in such preparations, sometimes quickly, sometimes slowly, opaque granules and minute needle-like crystals almost always become developed.

To avoid this Da Fano proposed (*Proc. Physiol. Soc. Journ. Physiol.*, liv, 1921) to treat sections much in the same way as by the so-called process of toning and fixing Bielschowsky preparations and the like. (See DA FANO, *ibid.*, liii, 1920.) He proceeds thus: Pieces which, by a trial section, have been found well impregnated, are washed for some hours in distilled water and then brought, through many changes of alcohol of ascending strengths, into absolute alcohol, and then imbedded in celloidin in the usual way. The celloidin blocks are hardened in 70 per cent. alcohol, where they can be safely left for many days and weeks. Sections of the desired thickness are collected in 60 per cent. alcohol, transferred into distilled water and here thoroughly washed. They are then treated for five to ten minutes with 5 per cent. ammonia and washed over again in two or three changes of distilled water. At this point toning is carried out by means of a slightly acidified 0·2 per cent. gold chloride solution, in which

sections are left for ten or fifteen or twenty minutes, according to their thickness. After a quick washing in distilled water they are passed for three to five minutes into 5 per cent. sodium hyposulphite and washed once more in distilled water. From this they are transferred successively into 30, 50, and 70 per cent. alcohols, to each of which 1 drop of saturated iodine tincture to every 5 c.c. of alcohol has been added. Sections remain in each alcohol ten to fifteen minutes and are lastly transferred into pure 70 per cent. alcohol.

At this point the process is ended, and one can proceed to mount the sections in the usual way, or re-transfer them into distilled water, counterstain them lightly with a carmine solution, dehydrate with alcohols of ascending strength up to 95 per cent., pass them through two changes of carbol-xylol and mount them under a thin cover-glass in xylol-colophonium or balsam. If desirable and safe, the celloidin can be removed before definite mounting by passing sections through absolute alcohol, and alcohol-ether if necessary.

The process is simpler than the rather complicated platinum substitutions of Robertson and Macdonald (*Journ. Ment. Sc.*, xlvii, 1901, p. 327) and is so quickly and easily carried out that many sections can be manipulated at the same time.

PROCESSES SIMILAR TO GOLGI'S METHODS OR SUITABLE FOR THE SAME PURPOSES

1045. Ziehen's **Gold and Sublimate Method** (*Neurol. Centrbl.*, x, 1891, p. 65). Small pieces of fresh tissues are put into a large quantity of a mixture of equal parts of 1 per cent. corrosive sublimate and 1 per cent. gold chloride, and left therein for at least three weeks, preferably for several months up to five, by which time they will have become of a metallic red-brown colour. They are then gummed to a cork or wooden cube and cut without imbedding. Sections are treated either with Lugol's solution diluted with 4 volumes of water, or with diluted tincture of iodine, until duly differentiated, then washed, dehydrated, and mounted in balsam. Both medullated and non-medullated nerve-fibres, as well as nerve-cells and neuroglia cells are stained.

1046. Krohnthal's **Lead Sulphide Impregnation** (*Neurol. Centrbl.*, xviii, 1889; *Ztschr. wiss. Mikr.*, xvi, 1899, p. 235). Pure formic acid is slowly added to a saturated solution of lead acetate till white crystals of lead formiate are abundantly formed. The mother liquid is filtered off, and the crystals are dissolved to saturation in distilled water. Equal volumes of this saturated solution of lead formiate and 10 per cent. formalin form the fixing fluid in which pieces of brain or spinal cord are left for five days. Tissues are then directly brought into a mixture of equal

parts of 10 per cent. formalin and sulphuretted hydrogen. After a few minutes the first discoloured portion of this mixture is poured off and replaced with fresh solution, in which pieces remain for another five days. They are then gradually dehydrated and imbedded in celloidin. Sections are cleared in carbol-xylol (1 : 1) and mounted in balsam under a cover. Nerve-cells and nerve-fibres are extensively impregnated.

Corning (*Anat. Anz.*, xvii, 1900, p. 108) hardens the tissues in 10 per cent. formalin and then brings them into the lead formiate which he buys from Merk. He prefers to cut without imbedding.

1047. Wolter's **Chloride of Vanadium Process** (*Ztschr. wiss. Mikr.*, vii, 1891, p. 471). Central or peripheral nervous tissues are fixed in Kultschitzky's solution, followed by alcohol as described in § 56. Celloidin sections, 5 to 10 μ thick, are mordanted for twenty-four hours in a mixture of 2 parts of 10 per cent. vanadium chloride and 8 parts of 8 per cent. aluminium acetate. They are then washed for ten minutes in water, stained for twenty-four hours in an incubator in Kultschitzky's hæmatoxylin, and differentiated in 80 per cent. alcohol acidified with 0·5 per cent. of hydrochloric acid until slightly blue-red. The acid is then removed by washing with pure alcohol, and the sections dehydrated, cleared with origanum oil, and mounted in balsam. Axis-cylinders, nerve-cells and glia cells are stained, the myelin being coloured only when the differentiation in the acid alcohol has been insufficient.

1048. Azoulay's **Ammonium Vanadate Process** (*Bull. Soc. Anat., Paris*, lxix, 1894, 5th S., p. 924). Wash in water thin sections of material fixed in a bichromate solution and imbedded in celloidin. Lift a section on a slide and pour on it a few drops of 0·5 per cent. ammonium vanadate, wait a moment, pour off the stain, wash with a little distilled water and pour on the section a few drops of 2·5 per cent. tannin. After a few minutes pour off the tannin solution, wash, and start all over again, and so on until axis-cylinder and nerve-cells are stained dark green. Wash quickly, dehydrate and mount. These preparations photograph well.

1049. Fajerstajn's **Hæmatoxylin** (*Poln. Arch. Biol. Med. Wiss.*, i, 1901, p. 189). Make sections, by means of the freezing microtome, of material fixed for two to seven days in 5 to 10 per cent. formalin. Transfer them into 0·25 to 0·5 per cent. chromic acid, and after twenty-four hours wash them well, and put them to stain for another twenty-four hours in 1 per cent. aqueous solution of hæmatoxylin. Differentiate by Pal's method.

1050. Nabias' **Method** (*C. R. Soc. Biol.*, lvi, 1904, p. 426). Sections of material fixed in alcohol-corrosive sublimate or any other fixing agent easily allowing the penetration of iodine are treated until yellow with Lugol's solution (Gram's formula).

They are then quickly washed and treated for a few minutes with 1 per cent. gold chloride, quickly washed once more, and reduced in 1 per cent. watery solution of anilin oil or resorcin. Dehydrate and mount in balsam.

1051. Lennhoff's **Processes** (*Neurol. Centrbl.*, xxix, 1910, p. 20). (1) *Polychrome-methylen blue and potassium sulphocyanide method for axis-cylinders:* Fixation not stated. Stain sections in polychrome methylen blue for two to five minutes, wash them in distilled water and transfer them for half to twenty-four hours into potassium sulphocyanide (strength not stated). Wash, dehydrate, clear, and mount in balsam. (2) *Polychrome-methylen blue and potassium ferricyanide method for axis-cylinders and nerve-cells:* Sections of material fixed in alcohol are treated as above, using potassium ferricyanide instead of the sulphocyanide. (3) *Iron method:* Sections are kept for thirty seconds in 2 c.c. of a 15 per cent. solution of tannin to which 3 drops of a 5 per cent. solution of oxalic acid have been added. Rinse them first in distilled water and then for a few seconds in 1 per cent. solution of iron chloride until no further blackening occurs. Wash, dehydrate and mount in balsam. Axis-cylinders black, nerve-cells grey.

Apáthy's **Gold Method.** See § 410.

Gerlach's **Bichromate and Gold Process.** See § 408.

Ramón y Cajal's **Gold Method.** See *Rev. trim. Micr.*, v, 1900, p. 95.

Upson's **Gold and Iron and Vanadium Methods.** See Mercier, *Ztschr. wiss. Mikr.*, vii, 1891, p. 474.

Magini's **Zinc Chloride Process.** See *Boll. Acc. med. Roma*, 1886, or *Ztschr. wiss Mikr.*, v, 1888, p. 87.

Monti's **Copper Process.** See *Rend. R. Acc. Lincei, Roma*, v, 1889, p. 705.

Strahuber's **Anilin Blue Method.** See *Centrbl. allg. Path.*, xii, 1901, p. 422.

Chilesotti's **Carmine Stain.** See *Centrbl. allg. Path.*, xii, 1902, p. 191; *Ztschr. wiss Mikr.*, xix, 1902, p. 161, and xx, 1903, p. 87.

Kaplan's **Anthracen Ink Method.** See *Arch. Psych.*, xxxv, 1902, p. 825.

Mallory's **Phosphomolybdic Hæmatoxylin.** See § 311.

Donaggio's **Tin Stain.** See § 313.

METHYLEN BLUE METHODS NOT CONSIDERED IN CHAPTER XVI.

1052. S. Meyer's **Method for the Central Nervous System** (*Arch. mikr. Anat.*, xlvi, 1895, p. 282, and xlvii, 1896, p. 734). The method consists essentially in injecting animals subcutaneously with large quantities of a solution of methylen blue B.X., and in treating the central organs (brains) with Bethe's fixing bath. S. Meyer used, at first, a 1 per cent. solution; later, a solution of methylen blue B.X. saturated at the body temperature of the animal to be injected (viz., about 5 to 6 per cent.). The injections are to be made at short intervals and in such a way that the animal receives the total quantity it can support in about one to two hours. A cat can support even 150 c.c.; half-grown rabbits, 30 to 50 c.c.; fully-developed guinea-pigs, 30 to

50 c.c.; new-born kittens, 15 to 25 c.c. As soon as the animal used is dead, the brain is removed, divided into two to four pieces, and these plunged in 10 per cent. ammonium molybdate to which 1 drop of HCl for every gram of ammonium molybdate is added. Here they remain for about twenty-four hours at 0° C. Pieces are then washed for two hours in running tap-water, passed quickly through the ascending series of alcohols into absolute alcohol, and, lastly, imbedded in paraffin in the usual way.

1053. Ramón y Cajal's **Diffusion Process** (*Rev. Trim. Micr.*, i, 1896, p. 123). The brain is exposed, and by means of a sharp razor the cortex is divided into slices about 2 mm. thick. The slices are then covered on both sides, either with finely powdered methylen blue or impregnated with a saturated solution of the same and replaced in their natural situation. The brain is covered over again with its case for about half an hour, after which the slices are removed and fixed for a couple of hours in Bethe's ammonium molybdate solution. They are then washed and hardened for three or four hours in a mixture of 5 parts of chloroplatinic acid, 40 parts of formalin, and 60 parts of distilled water. After another quick wash and a brief treatment (? a few minutes) with a 1 : 300 alcoholic solution of chloroplatinic acid, they are dehydrated and imbedded in paraffin. The sections may also be quickly treated with the same weak alcoholic solution of chloroplatinic acid, cleared with xylol or bergamot oil, and mounted in the usual way.

Catois' **Method for Fishes** (*C. R. Ac. Sc.*, cxxiv, 1897, p. 204). Small quantities (2 to 3 c.c.) of a concentrated solution of methylen blue, prepared with physiological salt solution, is injected into the branchial vessels or intramuscularly. The brain is removed after half an hour, divided into slices, and then left for another half hour in the same concentrated solution used for injecting the animal. The slices are then fixed in the usual ammonium molybdate solution, or in Cajal's chloroplatinic acid mixture.

See also the valuable account of Dogiel, *Methylen-blau zur Nervenfärbung* in the *Enzykl. mikr. Techn.*, 2nd ed., 1910, and the article of Gordon in *Anat. Rec.*, iv, 1910, p 267; and that of Michailow in *Ztschr. wiss. Mikr.*, xxvii, 1910, p. 1, in which the literature of the subject is critically discussed.

1053 *bis*. **Methods demonstrating Neurokeratin Network.** Platner (*Ztschr. wiss. Mikr.*, vi, 1889, p. 186) fixes for several days in a mixture of 1 part of Liq. Ferri Perchlor. (Ph.G., ed. 2) and 3 to 4 parts of water or alcohol, washes out well in water and stains for several days or weeks in a concentrated solution of "Echtgrün" (dinitroresorcin) in 75 per cent. alcohol. See also Beer, *Jahrb. Psychiatrie*, ii, 1893.

Cox (*Anat. Hefte*, i, 1898, p. 102, note) fixes nerves in 2 per cent. osmic acid (rabbit) or 1 per cent. (frog), washes, dehydrates, clears with bergamot oil, and mounts in balsam. The bergamot oil dissolves out the myelin, and leaves the neurokeratin visible. It may be necessary to leave the nerves for forty-eight hours in the oil.

Corning (*Anat. Anz.*, xvii, 1900, p. 309) studies the neurokeratin network in the sciatic of the frog by means of sections of sublimate material strongly stained with iron hæmatoxylin.

Kaplan (*Arch. Psychiatr.*, xxxv, 1902, p. 825) stains sections with acid fuchsin and differentiates them by Pal's method.

Gedoelst (*La Cellule*, v, 1889, p. 136) has the following: (*a*) A nerve is treated with liquid of Perényi, either pure or with addition of a trace of osmic acid, and examined in glycerin. By this treatment the myelin loses its excessive refractivity and the neurokeratin network comes out clearly. (*b*) Silver nitrate. Good images, but uncertain. (*c*) Treatment with a mixture of 1 per cent. osmic acid and absolute alcohol. The network comes out black.

CHAPTER XLII *

MYELIN STAINS

1054. Iron Hæmatoxylin. According to A. Bolles Lee (see 1913 ed.) the simplest way of staining myelin is to make paraffin sections of formol material and stain them with Heidenhain's iron hæmatoxylin exactly as for centrosomes (say, twelve to fourteen hours in the mordant, six in the hæmatoxylin, and a few minutes for the differentiation). Sections best not over 15 μ. One may counterstain the cells with carmalum, but not for more than half an hour, or the hæmatoxylin will be attacked. The stain is not so æsthetic as Weigert's, but quite as sharp. Axis-cylinders are not shown.

Regaud (*C. R. Acad. Sc.*, cxlviii, 1909, p. 861), but adding a *chrome mordantage* either concurrently with the formol fixation, or subsequently; Nageotte (*C. R. Soc. Biol.*, lxvii, 1909, p. 542); Houser (*Journ. Comp. Neurol.*, x, 1901, p. 65), and Spielmeyer (*Neurol. Centrbl.*, xxix, 1910, p. 348; and his *Technik d. mikrosk. Untersuch. d. Nervensystems*, 1924, p. 98), stain frozen sections of 25 to 35 μ with Heidenhain's iron hæmatoxylin. Loyez (*C. R. Soc. Biol.*, lxix, 1910, p. 511) makes celloidin sections of formalin fixed material, and after mordanting in iron alum, stains them in Weigert's lithium carbonate hæmatoxylin, preferably unripened, differentiates first lightly, till the grey matter begins to appear, in the iron alum, then washes, and differentiates further in Weigert's borax ferricyanide; Gilbert (*Ztsch. wiss. Mikr.*, xxviii, 1911, p. 279) mordants with iron alum, stains with *molybdic acid hæmatoxylin*, and differentiates with the borax ferricyanide; Stoeltzner (*ibid.*, xxiii, 1906, p. 329) mordants celloidin sections of formol material for five minutes in *Liq. ferri sesquichlorati*, stains in 0·5 per cent. hæmatoxylin, and differentiates in the mordant or in borax ferricyanide.

Weil ("Textbook of Neuropathology," 1934, Kimpton, London) uses frozen celloidin or paraffin sections from 20 to 30 μ thick. Frozen sections are placed in 70 per cent. alcohol for five to ten minutes and are then washed in distilled water before staining. The sections are stained at 55° C. for twenty to thirty minutes in equal parts of 4 per cent. iron alum and a 1 per cent. aqueous solution of hæmatoxylin prepared from a 10 per cent. alcoholic solution at least six months old. After differentiation first in

* Revised by J. G. G. and R. O. S.

iron alum and then in a solution of borax 10 grm., potassium ferricyanide 12·5 grm., distilled water 1000 c.c., the sections are washed three times in tap-water, a few drops of ammonia being added to the second washing water. If sections are over 30 μ in thickness, Weil mordants first in 5 per cent. potassium bichromate, washes twice in tap-water before staining, and after differentiation treats them with $\frac{1}{4}$ per cent. potassium permanganate and then, following a quick wash in tap-water, with a solution of oxalic acid 2·5 grm., sodium bisulphite 2·5 grm., distilled water 1000 c.c.

Olivecrona (*Centrbl. f. Allg. Path. u. Path. Anat.*, xxviii, 1917, p. 521) places frozen sections cut at 15 to 20 μ in 20 per cent. alcohol and then transfers them to 70 per cent. alcohol for ten minutes before staining for one hour in a freshly prepared mixture of 2 parts of a 1 per cent. solution of hæmatoxylin in 95 per cent. alcohol and 1 part of a solution containing liquor ferri perchloride 4 c.c., concentrated hydrochloric acid 1 c.c., distilled water 100 c.c. The sections are differentiated, after washing in tap-water, in liquor ferri perchloride 8 c.c., concentrated hydrochloric acid 1 c.c., distilled water 100 c.c. After washing in distilled water the sections are placed for fifteen minutes in water to which a few cubic centimetres of a concentrated aqueous solution of lithium carbonate have been added.

Loyez' and Weil's methods are now widely used in pathological anatomy. If a colourless background is desired, stain for one to two hours at 45° to 50° C., and differentiate by acid alcohol, or by Pal's oxalic acid-sodium sulphite mixture (*vide* § 1058).

Benda's **Rapid Method** (*Berlin klin. Wochenschr.*, xl, 1903, p. 748). Sections of *formol material* by the freezing process (alcohol being avoided) are stained (without any mordanting) for twenty-four hours in Boehmer's hæmatoxylin, differentiated with Weigert's ferricyanide, and mounted in balsam. Only recommended for peripheral nerves, or for preliminary examination of the central nervous system.

Nageotte (*C. R. Soc. Biol.*, lxv, 1908, p. 408) recommends fixing in 10 per cent. formalin to which is added from 1 to 7 per cent. sodium sulphate. The sections are cut by the freezing method, and stained for half an hour at 45° to 50° C. in Mayer's hæmalum, washed and differentiated in borax-ferricyanide.

Similarly the *Enzycl. mik. Technik.*, 1910, ii, p. 239, with *fresh material* cut by the freezing process, and the sections mounted in lævulose (as alcohol somewhat extracts the stain).

1055. Weigert's Methods. There have been in all three methods of Weigert: the 1884 method, the 1885 method, and the 1891 method.

The 1884 method (*Fortschr. d. Med.*, ii, 1884, pp. 120, 190; *Ztschr. wiss. Mikr.*, i, 1884, pp. 290, 564), which depends on the formation of a chrome lake of hæmatoxylin, may be considered as superseded. Not so the two others, which depend on the formation of a copper lake in addition to the chrome lake.

1056. WEIGERT'S **1885 Method** (*Fortschr. d. Med.*, iii, 1885, p. 236; *Ztschr. wiss. Mikr.*, 1885, pp. 399, 484; *Ergebn. Anat.* vi, 1896 (1897), p. 10) The tissues are hardened in potassium bichromate. WEIGERT takes (*Ergebn.*, p. 10) a 5 per cent. solution and if time is an object hardens in a stove. (Other bichromate mixtures will do, *e.g.* Müller's, Kultschitzky's, Zenker's; Erlicki's is not to be recommended.) The tissues are "ripe" for staining when the hardening has been carried to a certain point. They are first yellow, without differentiation of the grey matter from the white; these are unripe. Later they show the grey matter light brown, the white matter dark brown; and these are ripe.

More recently (*Ergebn.*, p. 14) he added to the bichromate solution 2 per cent. of chrome alum or of chromium fluoride, which hastens the hardening, so that small specimens become brown and ripe in four to five days, without stoving.

After hardening, tissues are generally imbedded in celloidin and the blocks hardened in the usual way. They are then put, for one or two days, in an incubating stove, into a saturated solution of neutral *copper acetate* diluted with 1 volume of water. By this treatment the tissues become green and the celloidin bluish-green. They may then be kept, till wanted for sectioning, in 80 per cent. alcohol.

Sections are made, well washed in water, and brought into a stain composed of :—

Hæmatoxylin	0·75 to 1 part.
Alcohol	10 parts.
Water	90 ,,
Saturated solution of lithium carbonate	1 part.

They remain there, for spinal cord, two hours; for medullary layers of brain, two hours; for cortical layers, twenty-four hours.

They are then again well washed with water, and brought into a decolourising solution composed of :—

Borax	2·0 parts.
Ferricyanide of potassium . .	2·5 ,,
Water	200·0 ,,

They remain there until complete differentiation (half an hour to several hours), and are then *well washed* with water (running, or changed several times), dehydrated, and mounted in balsam. They may be previously counterstained, if desired, with alum carmine.

The method is applicable to the study of peripheral nerves as well as to nerve-centres, and also the study of lymphatic glands, skin (see SCHIEFFERDECKER, *Anat. Anz.*, ii, 1887, p. 680), bile capillaries, and other objects.

The process is applicable to tissues that have been hardened in alcohol or in any other way, provided that they be put into a solution

of a chromic salt until they become brown before mordanting them in the copper solution.

It is not necessary that the mordanting be done in bulk. Max Flesch (*Ztschr. wiss. Mikr.*, iii, 1886, p. 50) prefers (following Lichtheim) to make the sections first, and to mordant them separately.

Vassale (*Riv. sperim. Freniatr.*, xv, 1889, p. 102) *first* stains the section in 1 per cent. hæmatoxylin for three to five minutes, then puts them for three to five minutes into saturated solution of copper acetate, and differentiates as Weigert.

1057. Weigert's **1891 Method** (*Deutsche med. Wochenschr.*, xvii, 1891, p. 1184). The material is hardened in bichromate and imbedded in celloidin (see last §). It is then (according to the latest form of the process (*Enzycl. mik. Technik.*, 1903, p. 942)), put for twenty-four hours in a stove into a solution of $2\frac{1}{2}$ parts of chromium fluoride, 5 of copper acetate, and 5 of acetic acid in 100 of water.*

Sections are then made and stained for from four to twenty-four hours at room temperature in a freshly-prepared mixture of 9 volumes of (A), a mixture of 7 c.c. of saturated aqueous solution of lithium carbonate with 93 c.c. of water, and 1 volume of (B), a solution of 1 grm. of hæmatoxylin in 10 c.c. of alcohol (A and B may be kept in stock, but A must not be too old). The sections should be loose ones, and not thicker than 25 μ. They are then washed in several changes of water, and treated with 90 per cent. alcohol, followed by carbol-xylol, or by a mixture of 2 parts of anilin oil with 1 of xylol, then pure xylol and xylol balsam (not chloroform balsam).

It was, however, found that preparations thus made, *without differentiation*, did not keep well, and Weigert (*Ergebn. Anat.*, iii, 1894, p. 21) reverted to the practice of differentiating with the borax-ferricyanide mixture.

Later still (*Enzycl. mik. Technik.*, 1903, p. 942) he employed a stain composed of equal parts of (A), a mixture of 4 c.c. of the officinal *Liquor ferri sesquichlorati* P. G. with 96 of water, and (B), a mixture of 10 c.c. of a 10 per cent. alcoholic solution of hæmatoxylin with 90 of 96 per cent. alcohol. The two (A and B) must be mixed immediately before use, and the sections should remain in the stain overnight or longer, then rinsed and differentiated as usual. This has the advantage of demonstrating very fine fibres, and of giving a *colourless background.*

For difficult objects the differentiating liquid may be diluted with water, and gives better results than dilute acetic or hydrochloric acids or the like, which were formerly recommended.

* Instead of the chromium fluoride one may use chrome alum, as Weigert did at one time, and as some still do. But then one must boil, as directed for Weigert's **Neuroglia stain.**

By means of Weigert's methods only the myelin sheaths of normal nerve-fibres are stained, whilst those of degenerated tracts are of a paler colour and, if the degeneration is sufficiently old, they may even be stainless. See also § 1081.

P. MEYER (*Neurol. Centrbl.*, xxviii, 1909, p. 353) hardened formol material in 5 per cent. potassium bichromate at 37° C. for from two weeks to several months, changing the mordant frequently, until the white matter was brown. After imbedding in celloidin, sections 50 μ thick are put first into Weigert's gliabeize for twenty-four hours at 37° C., then washed in 70 per cent. alcohol and stained for twenty-four hours in Weigert's iron hæmatoxylin. They are differentiated first with diluted borax-ferricyanide solution and then, if necessary, with a stronger solution.

For *Sheldon's* modification, which is also based on a formalin fixation see *Folia Neurobiol.*, viii, 1914, p. 1.

MODIFICATIONS OF WEIGERT'S METHOD

1058. PAL's **Method** (*Wien. med. Jahrb.*, N.F. i, 1886, p. 619; *Ztschr. wiss. Mikr.*, vi, 1887, p. 92; *Med. Jahrb.*, N.F. ii, 1887, p. 589). One proceeds as in WEIGERT's process, but *omitting the copper bath.* After staining in the hæmatoxylin solution, the sections are washed in water (if they are not stained of a deep blue a trace of lithium carbonate must be added to the water). They are then brought for 15 *to* 20 *seconds* into 0·25 per cent. solution of potassium permanganate, rinsed in water, and brought into a decolourising solution composed of :—

Oxalic acid	1·0 grm.
Potassium sulphite (SO_3K_2) . . .	1·0 ,,
Dist. water	200·0 c.c.

In a *few seconds* the grey substance of the sections is decolourised, the white matter remaining blue. If the differentiation is not complete the whole process can be repeated a second time, and so on. The sections should now be well washed out, and may be counterstained with Magdala red or eosin, or (better) with picro-carmine, acetic acid carmine, or alum carmine.

Pal's process gives brilliant results, the ground of the preparations being *totally colourless.* WEIGERT (*Ergebn. Anat.*, vi, 1896, p. 21) considered it superior to his own for thick sections, but not so safe for very fine fibres.

MARCUS stains by the Pal method sections of material hardened in formalin.

GUDDEN (*Neurol. Centrbl.*, xvi, 1897, p. 24) makes celloidin sections of material hardened in 5 to 10 per cent. formol followed by alcohol, treats them for ten hours with 0·55 per cent. chromic acid, rinses with water, and treats with 80 per cent. alcohol; then stains by the method of Pal. adding to the hæmatoxylin a few drops of dilute nitric acid (MINNICH).

TSCHERNYSCHEW and KARUSIN (*Ztschr. wiss. Mikr.*, xiii, 1896, p. 354) stain for twenty-four hours in KULTSCHITZKY's hæmatoxylin.

Pavlow (*ibid.*, xxi, 1904, p. 14) uses the permanganate twice as strong as Pal.

Kozowsky (*Neurol. Centrbl.*, xxiii, 1904, p. 1041) stains as Weigert, and differentiates the sections first with 1 per cent. permanganate, till the grey matter comes out brown, and finishes the differentiation with *Liq. ferri sesquichlorati.*

Pötter (*Ztschr. wiss. Mikr.*, xxvii, 1910, p. 238) fixes in 10 per cent. formalin, cuts slabs 15 mm. thick, and mordants these for fourteen days in Weigert's gliabeize, washing out in increasing strengths of alcohol and imbedding in celloidin. Thin sections, down to 10 μ, are stained for two and a half to three hours in Weigert's iron hæmatoxylin, *made without acid*, washed in distilled water, and differentiated, first in ½ per cent. pot. permanganate, and then in borax-ferricyanide solution.

Kaiser (*Neurol. Centrbl.*, xii, 1893, p. 364), Bolton (*Journ. Anat. and Phys.*, xxxii, 1898, p. 247), Wynn (*ibid.*, xxxiv, 1900, p. 381) and Laslett (*Lancet*, 1898, p. 321) use osmic acid either as a 1 per cent. solution or as Marchi's fluid as a mordant. Bolton also tried 2 per cent. ferric chloride, 2 per cent. iron alum, and ammonium molybdate as primary mordants on frozen sections with good results. For details see early editions.

1059. Kultschitzky's **Method** (*Anat. Anz.*, iv, 1889, p. 223; and v, 1890, p. 519). Specimens are hardened for one or two months in Erlicki's fluid, imbedded in celloidin or photoxylin, and cut. Sections are stained for from one to three hours, or as much as twenty-four, in a stain made by adding 1 grm. of hæmatoxylin dissolved in a little alcohol to 100 c.c. of 2 per cent. *acetic acid.* They are washed out in saturated solution of lithium or sodium carbonate. *Differentiation is not necessary*, but by adding to the lithium carbonate solution 10 per cent. of a 1 per cent. solution of potassium ferri-cyanide and decolourising therein for two or three hours or more, a sharper stain is obtained. After this the sections are well washed in water and mounted in balsam. Myelin dark blue.

Wolters (*Ztschr. wiss. Mikr.*, vii, 1890, p. 466) proceeds as Kultschitzky, except that he stains at 45° C. for twenty-four hours in 2 per cent. hæmatoxylin with 2 per cent. acetic acid, after which the sections are dipped in Müller's fluid, and differentiated by Pal's method.

Kaes (*Neurol. Centrbl.*, x, 1891, p. 456) stains in Kultschitzky's hæmatoxylin for two to three days at 45° C., and differentiates the sections by Pal's method in a porcelain dish, the bottom of which is perforated with fine holes.

Wolters' is now the standard " Weigert-Pal " method. It is usual to use material which has been fixed in formalin for not less than ten days, slices of which ⅓ to ½ cm. thick are placed direct in the mordant.

Perdrau transfers the sections after staining to a bowl of distilled water to which about 2 c.c. of a saturated solution of

lithium carbonate have been added. They are stirred about several times and transferred into a fresh bath of the same solution if necessary, until the celloidin is all but colourless. He lastly differentiates, as by Pal's method (§ 1058), washes, and counterstains either in alum carmine for ordinary work or in an alcoholic solution of eosin if the preparations are to be photographed.

1060. Mitrophanow (*Ztschr. wiss. Mikr.*, xiii, 1896, p. 470) mordants photoxylin sections for at least twenty-four hours at 40° C. in a mixture of equal parts of saturated aqueous solution of copper acetate and 90 per cent. alcohol, stains for ten minutes in Kultschitzky's hæmatoxylin, and differentiates with Weigert's ferricyanide fluid.

1061. Anderson (*Laboratory Journal*, v, 1922, p. 65, and Stitt's "Pract. Bact. Bloodwork and Parasit," 7th ed., London, 1923, p. 630) has modified Kultschitzky's method for frozen sections. He mordants sections for forty-eight to seventy-two hours at 37° C. in 90 c.c. of Weigert's fluorchrome-bichromate mordant with 10 c.c. of 2 per cent. calcium hypochlorite; transfers directly to Weigert's copper mordant for ten to thirty minutes, washes and stains for one hour at 50° C. in hæmatoxylin freshly made up in the following way. (Mix ½ grm. hæmatoxylin crystals in 10 c.c. of absolute alcohol, add 3 c.c. of 2 per cent. calcium hypochlorite, distilled water to 100 c.c. and then 3 c.c. of glacial acetic acid.) He transfers directly to Müller's fluid for ten to twenty minutes, washes well, and differentiates by Pal's method.

1062. Berkley's **Rapid Method** (*Neurol. Centrbl.*, xi, 1892, p. 270). Slices of tissue of not more than 2½ mm. in thickness are hardened for twenty-four to thirty hours in Flemming's fluid, at a temperature of 25° C., then in absolute alcohol, then imbedded in celloidin and cut. After washing in water the sections are put overnight into a saturated solution of copper acetate (or simply warmed therein to 35° to 40° C. for half an hour). They are then washed and stained for fifteen to twenty minutes in a lithium carbonate hæmatoxylin similar to Weigert's, warmed to 40° C., allowed to cool, and differentiated for one to three minutes in Weigert's ferricyanide liquid, which may be diluted if desired with one-third of water.

1063. Hill (*Phil. Trans.*, 184, b, 1894, p. 399) stains well-washed Müller material in bulk in *alum carmine*, cuts and mordants sections for twenty-four hours in half-saturated solution of copper acetate, stains and differentiates as Weigert, taking the differentiating fluid only half as strong.

1064. Streeter (*Arch. mik. Anat.*, lxii, 1903, p. 734) stains *small nerve-centres* in bulk (after mordanting in Weigert's bichromate and fluoride mixture) with Weigert's lithium carbonate hæmatoxylin (four to six days), washes for a couple of days in 70 per cent. alcohol, makes *paraffin sections*, and differentiates them by the method of Weigert or Pal.

1065. Besta's **Ammonio-Chloride of Tin Methods** (*Riv. Sperim. Freniatr.*, xxxi, 1905, p. 569). Pieces of peripheral nerves are fixed for one to three days in 100 c.c. of water with 25 of formol,

and 4 grm. of Merck's ammonio-chloride of tin, and then dehydrated and imbedded as usual. The sections may be stained in different ways : (*a*) For twenty-four hours in Mallory's phosphomolybdic-carbolic-acid hæmatoxylin with subsequent differentiation in Lugol's solution ; (*b*) for thirty to sixty minutes in a very diluted solution of Delafield's hæmatoxylin and then for a minute in Held's acetic solution of erythrosin ; (*c*) for five to ten minutes in erythrosin, and then for two hours in a mixture of equal parts of 1 per cent. hæmatoxylin and 4 per cent. ammonium molybdate with 3 drops of acetic acid to every 50 c.c. of the mixture.

1066. Gallein. ARONSON (*Centrbl. med. Wiss.*, xxviii, 1890, p. 577) stains sections of material, hardened in liquid of Erlicki or Müller and mordanted with copper acetate, for twelve to twenty-four hours in a solution of 3 to 4 c.c. of *gallein* in 100 c.c. of water with 20 of alcohol and 3 drops of a concentrated solution of sodium carbonate. Sections are differentiated by the method of Weigert, or Pal. Nerve-fibres red. A second stain with methylen blue may follow (best after differentiating with potassium permanganate). Similarly SCHRÖTTER (*Centrbl. allg. Path.*, xiii, 1902, p. 299).

1067. SCHRÖTTER (*Neurol. Centrbl.*, xxi, 1902, p. 338) also stains sections for two to three hours in a 5 per cent. solution of *sodium sulphalizarinate*, to which a few drops of 5 per cent. oxalic acid (enough to give an orange tint) are added, then differentiates until no more colour comes away in sodium carbonate solution of $\frac{3}{1000}$ strength, and mounts in balsam. Myelin red, on a colourless ground.

1068. Toluidine Blue and Methylen Blue. HARRIS (*Philadelphia Med. Journ.*, i, 1898, p. 897) stains sections (of material hardened as for Weigert's stain) for several hours in a 1 per cent. solution of *toluidine blue* in 1 per cent. borax solution, and differentiates in saturated aqueous solution of tannic acid. Similarly, but with *methylen blue*, in a complicated way FRAENKEL (*Neurol. Centrbl.*, xxii, 1903, p. 766).

BING and ELLERMANN (*Arch. Anat. Phys., Phys. Abth.*, 1901, p. 260) harden in 9 parts of acetone to 1 of formol, cut without imbedding, stain for five to ten minutes in saturated *methylen blue* solution, and put for one or two into saturated solution of picric acid.

1069. Other Modifications or Similar Methods. FLECHSIG, *Arch. Anat. Phys., Phys. Abth.*, 1889, p. 537 ; BREGLIA, *Ztschr. wiss. Mikr.*, vii, 1890, p. 236 ; ROSSI, *ibid.*, vi, 1889, p. 182 ; MERCIER, *ibid.*, vii, 1891, p. 480 ; HAUG, *ibid.*, p. 153 ; WALSEM, *ibid.*, xi, 1894, p. 236.

STRONG (*Journ. Comp. Neur.*, xiii, 1903, p. 291) finds *copper bichromate* (of 2 to 3 per cent.) the best mordant ; and that the mordanting is best done before bringing into celloidin. After staining, he treats for half a minute with 0·25 per cent. osmic acid and differentiates as Pal.

K. KOCH (*Berl. Klin. Wochenschr.*, li, 1914, p. 422) makes sections by the freezing method of formalin material imbedded in gelatin, and after staining with Weigert's iron hæmatoxylin, differentiates by Pal's method, and mounts in glycerin jelly.

1070. Staining Normal Medullary Sheaths by Osmic Acid (EXNER, *Sitzb. Akad. Wiss. Wien.*, lxxxiii, 1881, Abth. 3, p. 151 ; BEVAN LEWIS, *The Human Brain*, 1882, p. 105). A portion of brain, not exceeding a cubic centimetre in size, is placed in 1 per cent. osmic acid, and after five to ten days is cut (best without

imbedding). The sections are treated with ammonia (20 drops to 50 c.c. of water, which clears up the general mass of the brain substance, leaving the medullated fibres black. The preparations are not permanent, unless (RANVIER, *Traité*, 1 ed., p. 1086) they are fixed for a quarter of an hour in osmic acid vapour.

1071. GREENFIELD and CARMICHAEL (*Brain*, lviii, 1935, p. 483). found this method the most satisfactory for the finest myelin sheaths in small peripheral nerves. These do not need to be left in the osmic acid for more than twenty-four to forty-eight hours and after sectioning in paraffin or celloidin are mounted from 95 per cent. alcohol in Gurr's neutral-mounting medium.

AZOULAY'S **Osmic Tannic Acid Methods** (*Anat. Anz.*, x, 1894, p. 25). (A) Sections of old Müller material are put for five to fifteen minutes into a solution of 1 : 500 or 1 : 1000 of osmic acid, rinsed with water, and put for two to five minutes into a 5 or 10 per cent. solution of tannin, warming them therein over a flame till vapour arises, or in a stove at 50° to 55° C. Wash for five minutes in water, counterstain with carmine or eosin, and mount in balsam. If the sections are too thick it will be necessary to differentiate by Pal's process, or with *eau de Javelle* diluted with 50 volumes of water. (B) Material that has been in an osmic mixture (fluids of Flemming, or Marchi, or Golgi). Sections as before, then tannin bath, warming for three to ten minutes, the rest as before.

HELLIER and GUMPERTZ (*Ztschr. wiss. Mikr.*, xii, 1895, p. 385) give for peripheral nerves, and HELLER (*ibid.*, xv, 1898, p. 495) for central nervous system, the following method. Sections of Müller material are put into 1 per cent. osmic acid (twenty-four hours at 37° C. for peripheral nerves; ten to thirty minutes, at room temperature, for central nerve-fibres). They are treated with pyrogallic acid (a photographic developer will do) till the nerve-fibres are black, then with a violet-coloured solution of potassium permanganate till the sections become brown, then with 2 per cent. oxalic acid till they become yellow-green. Wash out well between each operation.

ROBERTSON (*Brit. Med. Journ.*, 1897, i, p. 651) hardens tissues in Weigert's gliabeize for ten days or longer, and cuts sections either by freezing or after imbedding in celloidin. He places these in 1 per cent. osmic acid in the dark for half an hour, then in 5 per cent. pyrogallic acid for half an hour, and differentiates by Pal's method. (This method was used exclusively for the sections reproduced in Bruce's "Atlas of the Spinal Cord.")

ORR (*Journ. Path. and Bact.*, vi, 1900, p. 387) stained the fine cortical fibres by fixing very thin slices of cortex for forty-eight hours in 2 per cent. osmic acid, 8 c.c. 1 per cent. acetic acid, 2 c.c., reducing the osmic acid in 10 per cent. formol, and imbedding in celloidin or paraffin.

FINOTTI (*Virchow's Arch.*, cxliii, 1896, p. 133) makes sections of material that has been in Müller's fluid for not more than a few weeks or months, and puts them for four to ten hours (in the dark) into a freshly-prepared mixture of 1 or 2 parts of 1 per cent. osmic acid, and 1 part of a concentrated solution of picric acid in one-third alcohol. For peripheral nerves; myelin (normal), black.

WITTMAACK (*Arch. Ohrenheilk*, lxi, 1904, p. 18) mordants till green (temporal bones) in 90 parts of Müller's fluid with 10 of formol and 3 to 5 of acetic acid, decalcifies with nitric acid and formol, treats sections

(paraffin or celloidin) for a few minutes with 2 per cent. osmic acid, and reduces in 5 per cent. pyrogallol.

1072. Iron. ALLERHAND (*Neurol. Centrbl.*, xvi, 1897, p. 727) puts sections of Müller material for fifteen minutes into warm 50 per cent. solution of *Liquor ferri sesquichlorati*, then for an hour or two into 20 per cent. tannin solution (old and brown). They are then differentiated by Pal's method, using, however, the liquids twice as strong.

An iron-alum process is described by STRONG in *Journ. Comp. Neurol.*, xiii, 1903, p. 291.

1073. Silver Nitrate. VESTARINI-CRESI (*Att. Accad. Med. Chir. Napoli*, l, 1896) hardens in formol, cuts thick sections, washes them with 40 per cent. alcohol, puts them in the dark into 1 per cent. solution of silver nitrate in 40 to 70 per cent. alcohol, then washes thoroughly.

Similarly, MOSSE (*Arch. mik. Anat.*, lix, 1902, p. 401), impregnating bichromic material with 1 per cent. solution of *argentamin*, and reducing in 10 per cent. pyrogallic acid, and differentiating by the method of PAL.

MYELIN AND AXIS-CYLINDER STAINS

1074. Methylen Blue. SAHLI (*Ztschr. wiss. Mikr.*, ii, 1885, p. 1) stains sections of tissue hardened in bichromate for several hours, in concentrated aqueous solution of methylen blue, rinses with water, and stains for five minutes in saturated aqueous solution of acid fuchsin. If now the sections are rinsed first with water, then for a few seconds in a 1 : 1000 alcoholic solution of caustic potash, and lastly brought into a large quantity of water, the stain becomes differentiated, axis-cylinders being shown coloured red and the myelin sheaths blue.

Or (*ibid.*, p. 49), the sections are stained for a few minutes or hours in :—

Water	40 parts.
Saturated aqueous solution of methylen blue .	24 „
5 per cent. solution of borax	16 „

then washed either in water or alcohol until the grey matter is distinctly differentiated from the white substance, cleared with cedar-wood oil, and mounted in balsam. Preparations similar to those obtainable by Weigert's method.

1075. Acid Fuchsin. FINOTTI (*Virchow's Archiv.*, cxliii, 1896, p. 133) stains strongly in Delafield's hæmatoxylin, then for a few seconds in concentrated solution of picric acid, then in 0·5 per cent. acid fuchsin, and treats lastly with alkaline alcohol.

OHLMACHER (*Journ. Exper. Med.*, ii, 1897, p. 675) stains sections for one minute with gentian violet in anilin water, then for a few seconds in a 0·5 per cent. solution of acid fuchsin in saturated solution of picric acid diluted with 1 volume of water, and differentiates with alcohol and clove oil.

KAPLAN (*Arch. Psychiatr.*, xxxv, 1902, p. 825) mordants for months in Müller, stains sections for a day or more in $\frac{1}{3}$ per cent. aqueous solution of acid fuchsin, rinses in water acidulated with HCl, and differentiates by the method of Pal.

1076. Safranin. ADAMKIEWICZ (*Sitzb. Akad. Wiss. Wien. Math. Naturw. Kl.*, lxxxix, 1884, Abth. 3, p. 245) stains sections of Müller material in concentrated solution of safranin, differentiates in alcohol and clove oil, brings back again into water, washes in water acidified with acetic acid, and stains in *methylen blue*. Myelin red, nuclei violet.

Similarly, CIAGLINSKI (*Ztschr. wiss. Mikr.*, viii, 1891, p. 19) and STROEBE (*ibid.*, x, 1893, p. 384), the former employing safranin followed by anilin blue, whilst the latter first stains with anilin blue, then

differentiates with alcohol containing a very little caustic potash, and counterstains with safranin.

1077. **Congo Red.** Nissl (*Ztschr. wiss. Mikr.*, iii, 1886, p. 398) stains for three days in Congo red (5 parts to 400 of water) and differentiates in alcohol with 3 per cent. of nitric acid.

1078. **Other Methods.** Röthig's **Vital-Scharlach VIII Counterstain** (*Neurol. Centrbl.*, xxxiii, 1914, p. 219, and xxxiv, 1915, p. 265). Sections stained and differentiated by Weigert-Pal's method are kept for twenty-four hours at room temperature in a counterstaining fluid consisting of 90 c.c. of distilled water and 10 to 20 c.c. of a solution of Vital-Scharlach VIII, saturated at room temperature. They are then washed in distilled water for fifteen minutes and differentiated in 70 per cent. alcohol for from one or two hours up to twenty-four, when the celloidin will be found to be colourless. After another wash in 96 per cent. alcohol, sections are mounted as usual. Nerve-cells and their processes, as well as axis-cylinders red, the latter being visible within the deep blue myelin sheaths.

The method does not succeed if the sections were previously treated with an osmic acid solution. Vital-Scharlach VIII may also be used as a general stain, in which case the finished preparations are similar to those obtainable by the usual carmine stains.

Paladino's *palladium chloride* methods; see *Rendic. R. Accad. Scienze, Napoli*, iv, 1891, p. 14; *Arch. Ital. Biol.*, xvii, 1892, p. 145, and xix, 1893, p. 26.

For Wolter's *vanadium chloride* process, see next chapter.

Zosin's *magenta red method*; see *Neurol. Centrbl.*, xxi, 1902, p. 207.

Perusini's *remarks and methods* for the study of the white substance of the spinal cord: see *Journ. Psychol. Neurol.*, xix, 1912, p. 61.

METHODS FOR DEMONSTRATING DEGENERATED NERVE-FIBRES AND THE PRODUCTS OF MYELIN DEGENERATION

1079. Among the most satisfactory methods of demonstrating degeneration of myelin in either the central or peripheral nervous systems, must be counted such fat stains as Scharlach R or Sudan III. These can only be used for formol fixed material and either on frozen sections or after imbedding in gelatin as recommended by Koch (*Berl. Klin. Wochenschr.*, li, 1914, p. 422). For anatomical work, however, Marchi's method or one of its modifications is to be preferred, as the degenerated fibres are stained black and stand out sharply against a yellowish background. Osmic acid, as in the Marchi method, undoubtedly stains some of the earlier products of myelin degeneration, which are not demonstrated with Scharlach, but it has several disadvantages. Among these are the difficulty of penetrating the blocks thoroughly with the osmic acid, the tendency for black granules to appear on any fibres,

which have been damaged post-mortem, by stretching or bending, and the lack of permanency of the preparations when mounted in Canada balsam. To avoid these troubles it is necessary to pay great attention to detail in carrying out the methods, and even then the results are sometimes disappointing. Failure of penetration may be avoided by using plenty of osmic acid on very thin blocks of tissue or on frozen sections, or by the addition of nitric or acetic acid as recommended by Vassale and Orr. Mounting direct from 95 per cent. alcohol in an alcoholic resinous medium, such as Gurr's neutral mounting medium or "euparal," which avoids strong fat solvents such as benzene, chloroform or xylol, renders the stain more permanent.

1080. Marchi's **Method** (*Riv. Sperim. Fren.*, xii, 1886, p. 50). *Small pieces* of nervous tissue are hardened for a week in Müller's solution, and then put for a few days into a mixture of 2 parts of Müller's solution and 1 part of 1 per cent. osmic acid. Sections are cut, best without imbedding, and mounted in balsam. The myelin sheaths of normal nerve fibres take a yellowish-brown colour, those of degenerated fibres a black one.

For a critical review of this method and its modifications, see Weigert (*Ergebn. Anat.*, vii, 1897 (1898), pp. 1—8); Matuszewski (*Arch. path. Anat.*, clxxix, 1905, p. 12); De Lange (*Le Nevraxe*, x, 1908, p. 83); and Lewy (*Fol. Neurobiol.*, ii, 1909, p. 471).

De Lange suggests putting pieces 3 to 4 mm. thick first for a few days into a mixture of 3 parts Müller's fluid + 1 part of 1 per cent. osmic acid, strengthening the osmic gradually up to equal parts of 1 per cent. osmic acid and of Müller's fluid.

Orr (*Journ. Path. and Bact.*, vi, 1900, p. 387) recommends original fixation for from two to four weeks in Müller's fluid, from which thin pieces are put into 2 per cent. osmic acid 8 c.c. + 1 per cent. acetic acid 2 c.c.

Vassale (*Arch. Ital. Biol.*, xxvii, 1897, p. 131) takes 75 c.c. of Müller's solution, 25 c.c. of 1 per cent. osmic acid, and 20 drops of nitric acid.

Teljatnik (*Neurol. Centrbl.*, xvi, 1897, p. 521), puts fresh pieces of brain 15 mm. thick into weak Marchi's fluid and then into a more concentrated solution.

Nissl (*Encycl. mik. Technik.*, ii, p. 248), holding that alcohol attacks the myelin, cuts without imbedding, and hurries sections through alcohol and bergamot oil into balsam.

Ramón y Cajal (*Trab. lab. Biol. Madrid*, ii, 1903, p. 93) has a complicated method of treating Marchi material.

Busch (*Neurol. Centrbl.*, xvii, 1898, p. 476), puts thin slices of formol fixed material directly into a solution of 1 part osmic acid, 3 parts of sodium iodate and 300 of water. This method gives the same results as Marchi's, except that the background remains

almost colourless. Penetration often appears to be more rapid, but irregular black granules are sometimes found throughout the tissue. This appears to be due to some impurity in the formalin or to too long fixation. It can be avoided by thorough washing of the material before it is placed in Busch's fluid or by refixation for a few days in Müller's fluid, thereafter washing out before treating with osmic acid.

SWANK and DAVENPORT (*Stain. Tech.*, x, 1935, p. 87), after fixing in 10 per cent. formalin for forty-eight hours, cut the tissues to be stained into blocks 3 mm. thick and place them in

1 per cent. potassium chlorate . . .	60 c.c.
1 per cent. osmic acid	20 ,,
glacial acetic acid	1 ,,
37 per cent. formaldehyde gas solution (Merck's reagent)	12 ,,

using about 15 volumes of reagent to 1 volume of tissue and staining for seven to ten days. The blocks are then washed in running water for twelve to twenty-four hours, dehydrated and imbedded in celloidin. After cutting, sections may be counter-stained in 1 per cent. aqueous cresyl violet for three to four minutes. They are washed in 70 per cent. alcohol and differentiated in acid alcohol or in equal parts of 95 per cent. alcohol and butyl alcohol, washed thoroughly in several changes of 95 per cent. alcohol, then passed through butyl alcohol and mounted in Canada balsam.

VENDEROVIC (*Anat. Anz.*, xxxix, 1911, p. 414) washes out the formalin thoroughly and lays slabs 2 to 5 mm. thick on several layers of filter paper in glass bottles which are then filled with Busch's fluid. The sections are occasionally turned over so that penetration may take place from both sides. Large pieces are kept in this a month or more, changing Busch's fluid occasionally. STEENSLAND (*Anat. Rec.*, viii, 1914, p. 123) recommends clearing sections of Marchi material with oleum origani cretici and mounting in chloroform-balsam. These methods which do not involve the use of Müller's solution, have the advantage that the tissue is less brittle and can be cut by the frozen section method.

Hurst has suggested a Marchi method for frozen sections which has been modified by R. C. J. STEWART (*Journ. Path. and Bact.*). He cuts frozen sections at 30μ from formalin fixed tissues. The sections are washed for one and a half hours in many changes of distilled water and are then placed in 2·5 per cent. potassium bichromate for twenty-four hours at 21° C. After washing in distilled water until the yellow colour has almost disappeared the sections are immersed in 1 per cent. osmic acid in the dark for sixteen to thirty-six hours at 21° C. (overnight usually suffices). They are then washed for five minutes in two changes of tap-water and are differentiated, if necessary, in 0·05 per cent. potassium permanganate, followed by Pal's solution, subsequently being

washed in many changes of tap-water, dehydrated and mounted in Gurr's neutral medium. Stewart recommends ringing the coverslips with Kronig's Deckglaskitt to prevent crystallisation of the mounting medium. (This may be prepared by gradually adding 8 parts of collophonium resin to 2 parts of melted paraffin wax of melting point 54° C., mixing thoroughly and allowing to cool.)

1081. Other Methods for demonstrating Degenerated Myelin. LORRAIN SMITH and MAIR (*Journ. Path. and Bact.*, xiii, 1909, p. 14) found that tissue which had been kept for an excessive length of time in strong chrome solutions gave, when treated by Weigert's myelin stain, the same picture as by the Marchi process, *i.e.*, only the degenerated myelin stained. They kept frozen sections for varying times up to twenty-four days at 37° C. in a saturated solution of pot. bichromate, later staining them with Kultschitzky's hæmatoxylin and differentiating with borax-ferricyanide.

Later (*Journ. Path. and Bact.*, xxiv, 1921, p. 364; xxv, 1922, pp. 143—403) they found that they could over-mordant sections in the same way with various aldehydes, and even with formaldehyde if it contained paraformaldehyde.

HURST (*Brain*, xlviii, 1925, p. 1) applied a similar method to the study of degenerating lipoids, mordanting frozen sections in Weigert's fluorchrome-bichromate mordant at 37° C. for varying periods from two to six days, and thereafter staining (one hour at 50° C.) and differentiating as Wolters. He found that myelin stains best after one or two days' mordanting and fatty acids after two or three days, whereas after four to six days' mordanting only neutral fat was stained.

LORRAIN SMITH (*J. Path. and Bact.*, xi, 1906, p. 415) stained fats in frozen sections with a mixture of basic anilin dyes and sulphurous acid. This method depends on the conversion of neutral fats to fatty acids which combine with the dye. R. C. J. STEWART (*Journ. Path. and Bact.*, xliii, 1936, p. 339) has successfully used the following modification: frozen sections of formalin fixed tissues are washed in tap-water for five minutes and placed in equal parts of 2 per cent. oxalic acid and 2 per cent. potassium sulphite for two to three minutes. They are then stained in 0·025 per cent. methylen blue for one hour and subsequently returned to the above bath for differentiation which should be controlled under the microscope; this is usually complete in six or seven minutes. The sections are again washed in tap-water, placed in 5 to 10 per cent. acid ammonium molybdate solution for three or four minutes and then transferred to a solution of equal parts of glycerin and saturated ammonium picrate in distilled water. They are mounted in glycerin jelly to which a little ammonium picrate has been added.

CHAPTER XLIII

NEUROGLIA AND SENSE ORGANS

NEUROGLIA *

1082. Introduction. Neuroglia cells may be isolated by teasing after maceration in weak solutions of potassium bichromate or 33 per cent. alcohol, and then stained, preferably by means of dilute picrocarmine or other carmine solutions. They may be studied, also, in sections made from non-imbedded material fixed in solutions of chromic salts and stained with carmine, nigrosin, orcein and so on. Sections made from either fresh material hardened by the ether freezing method and treated with a weak solution of osmic acid (§ 40), or from tissues hardened in potassium bichromate, can be advantageously stained with watery solutions of anilin-blue-black or nigrosin. Also, sections cut from material fixed, hardened and imbedded by the usual methods may, up to a point, be employed for getting a general, though incomplete, view of the amount and arrangement of the neuroglia in a given nervous organ. Iron hæmatoxylin, particularly after fixation in corrosive sublimate or other fluids containing it, gives good results with sections of central nervous organs of lower vertebrates, chiefly of fishes.

See GOLGI, *Opera Omnia*, i, pp. 1 and 3 to 70; ii, p. 461; RANVIER, *Traité*, etc.; BEVAN LEWIS, "*The Human Brain*"; E. MÜLLER, *Arch. mikr. Anat.*, lv, 1900, p. 11; STUDNICKA, *Anat. Hefte*, xv, 1900, p. 316, and the literature quoted therein.

But the best method for the study of morphology and relationship of ependyma cells and astrocytes has been for many years, and in a sense still is, Golgi's rapid process (§ 1028), the best material being that which has been placed for about two or three days in the osmio-bichromic mixture.

This method, however, does not allow of any tinctorial differentiation, either between neuroglia cells and nerve-cells, or between neuroglia cells and neuroglia fibres. One might even say that it is unsuitable for the demonstration of the latter, the existence of which was clearly established only after the publication of WEIGERT's method (see next §), the first and, perhaps even now, most important of all so-called specific processes for staining neuroglia fibres.

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But the Weigert method, whilst staining neuroglia fibres and nuclei of neuroglia cells intensely and, up to a point, specifically, leaves the cell-bodies of the latter entirely unstained. It consequently led to the erroneous conclusion that the processes of neuroglia cells were one and the same thing as the neuroglia fibres shown by the new method, and that the latter were, in the adult state, only contiguous to—viz., independent of—the cell body.

Efforts were, therefore, made to discover new methods suitable for the study of neuroglia fibres and neuroglia cells and their reciprocal relations. .Many modifications of Weigert's neuroglia stain, the methods of BENDA, MALLORY, ANGLADE and MOREL, HELD, RUBASCHKIN, DA FANO, etc., may be considered as the direct outcome of such efforts.

None of these methods, however, was sufficient to entirely solve the problems resulting from Weigert's discovery and from the comparison between the results attainable by the new neuroglia stain and Golgi's process.

Of these the most obvious difficulty arose from the fact that many neuroglia cells were stained in the cerebral cortex by Golgi's method which were not revealed by Weigert's stain. Ramón y Cajal in 1913 published his gold-sublimate method which stained all the neuroglia cells electively and revealed the structure of the cortical cells as well as those which Weigert's method showed. It proved that the cortical neuroglia cells differed in structure from the majority of the neuroglia cells found elsewhere in the nervous sytem. The term "*protoplasmic neuroglia*" was then applied to these cells, who do not contain the fibres demonstrated by Ranvier and Weigert, and the term "*fibrous neuroglia*" to those which do.

Other methods published by Achúcarro and Del Río-Hortega confirmed Cajal's work, but revealed, in addition, the processes of two other cell forms, which older writers had called "satellite cells" or "undifferentiated glia cells." There is now general agreement on the distinction of these two cell types, one of which, the "*oligodendroglia*" or "interfascicular glia," is probably a true neuroglial cell, whereas the other, the "*microglia*," appears to be mesodermal in origin, and to be closely related to the reticulo-endothelial system of Aschoff.

It may be said at once that none of these cells except the *fibrous neuroglia* cells can be stained in their entirety by any anilin stain. For their demonstration the only methods so far successful have been impregnations with metals such as silver, gold or platinum. Ford Robertson appears to have been the first to stain the oligodendroglia by a platinum method (*Scottish Med. & Surg. Journ.*, 1899). Further modifications of these methods have demonstrated rounded bodies or gliosomes on the cell-

bodies and processes of the *fibrous*, *protoplasmic* and *interfascicular* glia, but not on the *microglia*.

For minute technical details and some of the less commonly used methods, the original papers quoted in the following paragraphs should be consulted as well as NISSL (*Enzykl. mikr. Techn.*, ii, 1910, pp. 280 to 283); BONOME (*Atti R. Inst. Veneto Sc.*, lxvii, 1909).

METHODS FOR FIBROUS NEUROGLIA

1083. WEIGERT'S **Neuroglia Stain** (WEIGERT'S *Beitr. zur Kenntniss d. norm. mensch. Neuroglia*, Frankfurt-a.-Main, 1895; and the article "*Neurogliafärbung*" in *Enzykl. mik. Technik.* ii, 1910). Pieces of very fresh tissue of not more than $\frac{1}{2}$ cm. in thickness are put, for at least four days, into 10 per cent. formol. They are then mordanted for four or five days at 36° to 37° C. (or for at least eight days at the temperature of the laboratory) in a solution containing 5 per cent. of neutral copper acetate, 5 per cent. of acetic acid, and $2\frac{1}{2}$ per cent. of chrome alum, in water. "*Gliabeize.*"—(Add the alum to the water, raise to boiling point, and add the acetic acid and the acetate, powdered, or, instead of chrome alum, take chromium fluoride, which obviates the necessity of boiling.) If preferred, the mordant may be dissolved in the formol solution, so that the hardening and mordanting are done at the same time.

After mordanting, the tissues are washed, dehydrated, imbedded in celloidin, and cut. The sections (not too thick) are treated for ten minutes with a $\frac{1}{3}$ per cent. solution of potassium permanganate and well washed in water. They are then treated for two to four hours with a solution of "chromogen." This is a naphthaline compound prepared by the Hoechst dye manufactory. The solution to be used is prepared as follows: 5 per cent. of "chromogen" and 5 per cent. of formic acid (*of* 1·20 *sp. gr.*, about four times as strong as the officinal) are dissolved in water, and the solution carefully filtered. To 90 c.c. of the filtrate, 10 c.c. of a 10 per cent. solution of sodium sulphite are added.

After this the sections are put till the next day into a saturated (about 5 per cent.) solution of "chromogen." (According to Bolles Lee, Pal's potassium sulphite may be used instead of the "chromogen.")

They are next carefully washed and stained. This is best done on the slide. The stain is a warm-saturated solution of methyl violet in 70 to 80 per cent. alcohol (to which, after cooling and decanting, there may be added, if desired, 5 per cent. of a 5 per cent. aqueous solution of oxalic acid). The sections are treated with this for from a few seconds to one minute, and mopped up with blotting-paper, then treated for an instant with saturated solution of iodine in 5 per cent. potassium iodide.

They are then differentiated till clear and light blue with a mixture of equal parts of anilin oil and xylol, washed thoroughly with pure xylol, and mounted in balsam or, preferably, in turpentine-colophonium.

Glia fibres and nuclei blue, cytoplasm stainless.

This method *only* gives good results with the *human* subject.

SPIELMEYER (*Tech. der mik. Unter. des Nervensystems*, Berlin, 1924) recommends using a stain composed of 5 parts of a saturated alcoholic solution of methyl violet, 10 parts of absolute alcohol, and 85 parts of 5 per cent. phenol.

1084. Modifications of WEIGERT'S Method. See also MALLORY (*Journ. Exper. Med.*, 1897, p. 532).

STORCH (*Virchow's Archiv.*, clvii, 1899, p. 127).

BARTEL (*Ztschr. wiss. Mikr.*, xxi, 1904, p. 18).

WIMMER (*Centrbl. allg. Pathol. u. Pathol. Anat.*, xvii, 1906, p. 566).

GALESESCU (*C. R. Soc. de Biol.*, lxv, 1908, p. 429) fixes tissue first for five hours in 6 per cent. sublimate and then for forty-eight hours at 37° C. in 3 parts of Fol's modification of Flemming's chromi-osmo-acetic acid with 1 part of 7 per cent. sublimate, changing the fluid two or three times. After washing for two hours in running water the pieces are passed first for twenty-four hours into acetone to which a little tincture of iodine is added, then into pure acetone and imbedded in paraffin. Sections 3 to 4 μ thick, are put into a 5 per cent. solution of methyl violet 5B, in 80 per cent. alcohol, adding to each 20 c.c. of this stain 1 c.c. of 5 per cent. oxalic acid. Stain first in the cold for ten minutes and then warm gently over the flame for five minutes. Pour on Gram's iodine, heating again slightly, blot thoroughly with filter paper and decolourise with equal parts of xylol and anilin oil.

ANGLADE AND MOREL'S VICTORIA BLUE METHOD (*Rev. Neurol.*, ix, 1901, p. 157). Harden the tissue as Galesescu, and stain paraffin sections in a saturated aqueous solution of Victoria blue, heated till it steams; rinse with Gram's fluid, and differentiate, without washing, in xylol 1 part, anilin 2 parts. Wash well in xylol and mount in balsam. Simple, applicable to lower animals and gives very sharp pictures.

LHERMITTE and GUCCIONE (*Semaine Med.*, xxix, 1909, p. 205) have the following modifications of Anglade and Morel's method. Sections made by the freezing method from formalin material are collected in distilled water and then kept for two hours in a cold saturated solution of sublimate and for two days in a mixture consisting of 3 parts of 1 per cent. osmic acid, 35 of 1 per cent. chromic acid, 7 of 2 per cent. acetic acid, 55 of distilled water.

A slide is then covered with cigarette paper and a section floated on to this out of water, drained almost dry and stained for a few minutes with a 1·5 per cent. watery solution of Victoria blue, heating it gently till steam rises three to five times. Throw off excess of stain and pour on Lugol's iodine (iodine 1, pot. iodide 2, water 200) and leave on one minute. Pour off excess of iodine, pour on equal parts of anilin oil and xylol, and leave on till the heavier masses of stain are removed. Then turn the cigarette paper upside down on to a clean slide, blot firmly, and remove paper, leaving the section on the slide. Continue differentiation with anilin oil-xylol, wash thoroughly with xylol. Mount in Canada balsam.

Similarly DE ALBERTIS (*Pathologica*, xii, 1920, p. 240).

SPIELMEYER (*Technik der mik. Untersuch des Nerven*, Berlin, 1924) also gives the following "Heidelberger" method. Celloidin sections

of formalin, or better alcohol-fixed material are freed from celloidin and fixed to slides with methyl alcohol and stained for twelve hours in 1 per cent. Victoria blue, and further treated with iodine, etc., as in Weiger's method.

Merzbacher (*Journ. Neurol. Psych.*, 1909, xii, p. 1) treats either frozen or paraffin sections of formalin material or celloidin sections fixed to the side by methyl alcohol for two to five minutes with an alkaline alcoholic solution (absolute alcohol 70, 10 per cent. caustic soda 20, distilled water 10), stains in the cold for twenty-four hours with saturated solution of Victoria blue, boiled for one hour, treats with iodine and differentiates as Weigert.

Anderson (*J. Path. & Bact.*, xxvi, 1923, p. 431) treats frozen sections of formalin material or paraffin sections of material fixed, or after-hardened, in Bouin's fluid for ten to thirty minutes with a mordant consisting of 1 part of 5 per cent. ferric chloride and 2 parts of the following mixture. (Distilled water, 100 c.c., sod. sulphite 5 grm., oxalic acid 2·5 grm., pot. iodide 5 grm., iodine 2·5 grm., glacial acetic acid 5 c.c.) Wash in distilled water and transfer first to $\frac{1}{4}$ per cent. permanganate for five minutes and then to freshly-mixed Pal's solution for five minutes or more. The sections should be left in this solution until they can be stained. After a quick wash the sections are floated on an albuminised slide, blotted and almost allowed to dry. They are then stained by pouring on boiling 1·5 per cent. Victoria blue, and kept slightly warm in this stain for five minutes. After pouring off the stain strong Lugol's iodone (iodine 1 grm., pot. iodide 2 grm., distilled water 100 c.c.) is dropped on to the middle of the section and allowed to act for one minute. The section is then differentiated with equal parts of anilin oil and xylol for fifteen seconds, and then washed *till clear*, with xylol, after which it is blotted and further differentiated with xylol and anilin oil, washed thoroughly with xylol, and mounted in Canada balsam. Anderson points out that the anilin oil-xylol mixture is much more active so long as any water is left in the tissue and may easily differentiate the finer fibres too far at this stage, but once the water has been eliminated there is much less danger of over-differentiating.

Holzer (*Zeit. f. d. ges. Neur. u. Psych.*, lxix, 1921, p. 354) treats frozen sections of formalin material first for half to one and a half minutes with a mixture of 1 part of $\frac{1}{2}$ per cent. phosphomolybdic acid and 2 parts of 96 per cent. alcohol, floats them on to a slide out of this solution, blots with filter paper wetted with a mixture of 2 parts absolute alcohol, and 8 parts of chloroform, and stains with a solution of 0·5 grm. crystal violet in 2 c.c. of alcohol and 8 c.c. of chloroform. Wash off the stain with 10 per cent. watery pot. bromide solution. Blot once with filter paper wet with a mixture of 4 c.c. anilin, 6 c.c. chloroform, and 1 drop of 1 per cent. HCl, and differentiate further with this solution. Wash thoroughly with xylol and mount in balsam.

Warkany (*Zeitschr. f. Wissenschaft. Mikro.*, iv, 1924, 1, p. 508), treats frozen sections with equal parts of 1 per cent. phosphomolybdic acid and 95 per cent. alcohol, stains them as Holzer, but differentiates in two changes of anilin oil.

See also Aguerre, *Arch. mik. Anat.*, lvi, 1900, p. 509 ; Krause, *Abh. k. Akad. Wissench. Berlin. Anhang*, 1899.

Rubaschkin (*Arch. mik. Anat.*, lxiv, 1904, p. 577) recommends injecting centres of small mammals with the fixing liquid. To make this, take 100 parts of 2·5 per cent. solution of potassium bichromate and 0·5 to 1 of copper acetate, boil, and add 2·5 to 5 of glacial acetic acid. To this (which may be kept in stock) add, just before use, 10 per cent. of formol. Inject warm, and after ten minutes dissect out and

harden in the same fluid for five to seven days at 35° to 40° C. Dry superficially, put for six to twelve hours into 95 per cent. alcohol and imbed in celloidin or paraffin. Stain sections on the slide for six to twelve hours in saturated aqueous solution of methyl violet B; treat for half a minute to a minute with Gram's iodine in iodide of potassium; differentiate in anilin or clove oil, and pass through xylol into balsam. The method gives very sharp results with small mammals.

1085. Benda's **Method** (*Neurol. Centrbl.*, xix, 1900, p. 796; and his article "*Neurogliafärbung*," *Enzykl. mik. Technik*, ii, 1910, p. 308) is as follows:—The material is to be fixed in 90 or 93 per cent. alcohol for no less than two days. Pieces, not thicker than ½ cm. are put for twenty-four hours into officinal nitric acid 1 part, and distilled water 10 parts; for another twenty-four hours in 2 per cent. potassium bichromate; for forty-eight hours in 1 per cent. chromic acid. After washing for twenty-four hours, they are dehydrated in alcohols of ascending strength, cleared first in creosote (twenty-fours), then in benzol (twenty-four hours), and lastly imbedded slowly in paraffin, this being dissolved in benzol to saturation first at room temperature, then successively at 38°, 42° and 45° C., so that pure paraffin, melting at 58° C., is used only for the imbedding proper.

The sections, stuck to slides, are mordanted for twenty-four hours in 4 per cent. iron alum or in 50 per cent. *Liquor ferri sulfurici oxydati* P.G. thoroughly washed, put for two hours into an amber-yellow aqueous solution of sodium sulfalizarinate as directed in § 697, rinsed with tap-water, and put to stain in 0·1 per cent. toluidine blue either for fifteen minutes by warming until vapour arises, or for twenty-four hours at room temperature. After rinsing in 1 per cent. acetic acid or in a very dilute solution of picric acid, the sections are dried with filter paper, passed through absolute alcohol, and differentiated for about ten minutes with creosote. They are then dried once more with filter paper, washed with xylol and mounted in balsam.

Besides this, *Benda* recommends hardening and making paraffin sections as above, then staining by Weigert's method (§ 1083), but without passing the sections through the saturated solution of "chromogen," and using instead of Weigert's methyl violet solution a freshly-prepared mixture of 1 volume of saturated solution of crystal violet, 1 volume of 1 per cent. acid alcohol, and 2 volumes of anilin water.

Benda also uses Heidenhain's iron hæmatoxylin to stain paraffin sections of pieces treated as described, differentiating either with 2 per cent. iron alum or with Weigert's borax-ferricyanide mixture.

1086. Mallory's **Hæmatoxylin Stains** (*Journ. Exper. Med.*, v, 1900, p. 19). Zenker fixed tissues are cut in paraffin and after treatment with iodine and hyposulphite are put for a quarter of an hour into 0·5 per cent. solution of potassium permanganate, washed and put for another quarter of an hour into 1 per cent. solution of oxalic acid, well washed and stained for twelve to twenty-four hours or more in Mallory's *phosphotungstic hæmatoxylin.* Wash, dehydrate in 95 per cent. alcohol, clear with origanum oil, mount in xylol-balsam. Axis-cylinders and nerve-cells pink, neuroglia fibres and myelin blue. To get a more isolated stain of neuroglia, the sections should be brought for five to twenty minutes, after staining, into a 30 per cent. alcoholic solution of iron sequichloride. Neuroglia, fibrin and nuclei blue, the rest colourless.

(MALLORY's *phosphomolybdic hæmatoxylin* may also be used for the stain, but it is less elective.)

GREENFIELD (in Anderson's *How to Stain the Nervous System*, E. and S. Livingstone, Edinburgh, 1929), uses this method for celloidin sections of formalin fixed material. These are treated with 5 per cent. mercuric chloride for half an hour, followed by strong iodine for three minutes, and after a quick rinse in tap-water, by 5 per cent. hyposulphite for three minutes. The sections are then placed in ¼ per cent. potassium permanganate for five minutes, washed quickly in tap-water and transferred to 5 per cent. oxalic acid for five minutes. They are then washed in distilled water for ten minutes before being stained for twelve to twenty-four hours in Mallory's phosphotungstic hæmatoxylin. Differentiation in alcoholic ferric chloride is specially useful here.

KERNOHAN (*Am. J. Clin. Path.*, 1, 1931, p. 399) also uses formalin fixed material. He first washes fixed tissue for twenty-four hours in running water, then places it for four days in Weigert's primary myelin mordant and for two days in Weigert's secondary mordant (see §1057), subsequently imbedding in paraffin.

1087. DA FANO's Methods (*Ricerche Lab. Anat. Roma ed altri Lab. Biol.*, xii, 1906). *Method I.* is a modification of MALLORY's phosphotungstic hæmatoxylin process (§ 1085). Small pieces of fresh tissue are fixed for twenty-four to forty-eight hours in a mixture of 72 volumes of pyridine and 28 of 50 per cent. nitric acid. After washing for about six hours, the pieces are dehydrated and imbedded in paraffin. The sections, stuck to slides by the albumen method, are treated as by MALLORY's method, and stained with an old solution of MALLORY's phosphotungstic hæmatoxylin, *but prepared without the addition of hydrogen peroxide*. In order to increase the contrast between neuroglia fibres (blue-violet) and the protoplasm of neuroglia cells (pink) DA FANO dehydrates the stained sections in 95 per cent. alcohol to which a small quantity of an alcoholic solution of eosin has been added.

Method II. is a modification of BENDA's process (§ 1085). Very small pieces are fixed for thirty-six to seventy-two hours in a mixture of 2 volumes of the fixing fluid used for Method I. and 1 volume of 1 per cent. osmic acid. After washing for six to twelve hours, the pieces are imbedded in paraffin. The sections, stuck to slides, are successively mordanted for twenty-four hours each with WEIGERT's copper acetate-chromium fluoride fluid (§ 1083), 2 per cent. chromic acid, and 2 per cent. iron alum, rinsing in water before passing them from one into the other mordant. They are lastly either treated and stained as by BENDA's alizarine-toluidine blue process, or as by HEIDENHAIN's iron hæmatoxylin method.

Method III. was arrived at in an endeavour to make use of unsuccessful preparations made by CAJAL's reduced silver method. Pieces treated as by Cajal's formula 1a or one of its modifications (§ 1011), or simply fixed in 2 or 3 per cent. silver nitrate at 36° to 37° C., are imbedded in paraffin. The sections, stuck to slides, are bleached by Pal's differentiation method for myelin stain, and then mordanted and stained as by Method II.

1088. HELD's Method for Marginal Neuroglia (*Monatschr. Psych. Neurol.*, xxvi, 1909; *Ergänzungsh.*, p. 360). Tissues are preferably fixed by means of a modified Zenker's fluid consisting of Müller's fluid

100 c.c. and sublimate 3 grm., with the addition at the moment of use of acetic acid 3 c.c., formalin 0·5 c.c. The fluid should be warmed at 35° to 40° C. and injected through the blood-vessels, the blood being first washed away by means of Ringer's solution to which 1 : 1000 of amylnitrite was added. The tissues are treated in the usual way and imbedded in celloidin. The sections are first treated for five minutes with a 1 per cent. solution of caustic soda in 80 per cent. alcohol and washed in distilled water, and then mordanted for a few minutes in 5 per cent. iron alum and washed once more. For staining, Held adds to some distilled water a few drops of a very old molybdic acid hæmatoxylin, enough to impart to the water a bluish-violet tone, and stains therein for twelve to twenty-four hours at 50° C. The stain is prepared by dissolving 1 grm. of hæmatoxylin in 100 c.c. of 70 per cent. alcohol and adding an excess of molybdic acid. Differentiation is carried out by means of the same iron-alum solution used for mordanting ; wash well ; counterstain with v. Gieson picro-fuchsin solution ; wash in 96 per cent. alcohol, dehydrate and mount as usual.

Neuroglia cells and fibres greyish-black ; marginal neuroglia (*membrana limitans marginalis* and *membrana limitans perivascularis*) sharply differentiated ; connective tissue pink-red. Mallory's connective tissue stain (acid fuchsin-anilin-blue-Orange G), and to a less extent Masson's trichromic and M. Heidenhain's "Azan" stain (see § 811), give useful neuroglia pictures.

1089. Jakob (*Nissl-Alzheimer's Arb. u. d. Grosshirnrinde*, v, 1913) makes thin frozen sections of material fixed in gliabeize + 10 per cent. formol, washes rapidly in distilled water, and stains first for three to ten minutes in 1 in 1000 watery solution of acid fuchsin, and then after washing puts into saturated watery solution of phosphomolybdic acid for one to twenty-four hours. The sections are then, after another short wash, put into a mixture containing 0·5 grm. of water-soluble anilin blue, 2 grm. of Orange G, 2 grm. of oxalic acid and 100 c.c. of distilled water, for half an hour After a short wash in distilled water the sections are differentiated in 96 per cent alcohol, keeping them moving in this, until no more clouds of stain come off. They are then treated with absolute alcohol and xylol and mounted in balsam. (Glial cells and processes violet, axis-cylinders blue, myelin sheaths golden with slight rose tint. Ganglion cells and vessel walls dark blue, nuclei and blood corpuscles light red.)

Anderson ("*How to Stain the Nervous System*," E. and S. Livingstone, Edinburgh, 1929), has modified this method for paraffin and celloidin sections. Tissue to be imbedded in paraffin is best fixed in Bouin's or Zenker's fluid, but if already fixed in formalin should be placed in Bouin's fluid for sixteen to twenty-four hours before imbedding. This treatment is not necessary for material to be imbedded in celloidin. After washing in distilled water sections are placed for half an hour in the following mordant: iron alum, 1 grm.; sulphuric acid, 1 c.c.; 50 per cent. alcohol, 98 c.c. The sections are then washed in distilled water and stained for one to five minutes in ½ per cent. acid fuchsin in ½ per cent. glacial acetic acid. After a prolonged wash in distilled water they are transferred to 4 per cent. phosphomolybdic acid for thirty to forty-five minutes, washed again in distilled water and placed in Mallory's anilin-blue-Orange G. mixture for fifteen minutes. Paraffin sections are differentiated in absolute alcohol, celloidin sections in 95 per cent. alcohol. They are cleared in xylol and mounted in acid balsam.

Bailey (*Journ. Med. Res.*, xliv, 1923, p. 73) cuts thin paraffin sections of Zenker or Bouin fixed material and after treatment with iodised alcohol mordants for three days in 3 per cent. pot. bichromate, then

rinses and places in a neutral solution of ethyl violet and orange G for twelve hours. Blot and then agitate quickly in anhydrous acetone, place in toluol for a few seconds, then flood the slide with pure clove oil and differentiate further in 3 parts of clove oil and 1 part of 95 per cent. alcohol, rinse in pure clove oil, then toluol, xylol (six changes) balsam. (The neutral ethyl violet-orange G solution is most easily prepared by adding to 100 c.c. of distilled water 0·5 grm. of orange G and 1 grm. ethyl violet. Stir thoroughly and place in the oven to precipitate for twelve to twenty-four hours. Decant the supernatant liquid and wash the precipitate several times with distilled water. Place in the oven to dry. Make a saturated alcoholic solution of the dried powder as stock and use 1 part of this with 3 parts of 20 per cent. alcohol as staining solution.)

Bailey also uses this method on material fixed in Regaud's solution to demonstrate certain special granules in the cells of the pars anterior hypophysis.

Kultschitzky's **Rubin Method** (*Anat. Anz.*, vii, 1893, p. 357) is no longer used. For the slight modification of this method of Popow, see *Ztschr. wiss. Mikr.*, xiii, 1896, p. 358, and for that of Burchardt, *La Cellule*, xii, 1897, p. 364.

The method of Yamagiwa (*Virchow's Arch.*, clx., 1900, p. 358) is also no longer used.

1090. Methods for Neuroglia Cell-bodies and Granules. Oppenheim (*Neurol. Centrbl.*, xxvii, 1908, p. 643) mordants sections made from frozen formalin material with Weigert's copper acetate-chromium fluoride mixture and then stains them with Weigert's iron hæmatoxylin prepared without hydrochloric acid. An important point of this method is that the material and the sections should *not* have been treated with alcohol before staining.

Eisath (*Monatschr. Psych. Neurol.*, xx, 1906, p. 3 ; *Arch. f. Psych.*, xlviii, 1911, p. 897) fixes large pieces in a modified Orth's formol-Müller mixture consisting of water 1000 c.c., potassium bichromate 25 grm., sodium sulphate 15 grm., and formalin 150 c.c. to be added at the moment of using the mixture. After about four weeks the tissues are ready for being cut without imbedding, but can be kept for many months, and even years, in 4 per cent. formalin. The sections are collected in 4 per cent. formalin, in which they may be kept until wanted. For the staining the sections are put for thirty seconds in a 0·2 per cent. solution of sublimate, well washed in water, and lifted on to the slide, a dilution of an old Mallory's phosphomolybdic-carbolic acid hæmatoxylin being poured on them. After a few minutes they are washed with water, differentiated with a mixture of equal parts of 40 per cent. tannic acid, 50 per cent. alcohol, and 20 per cent. pyrogallic acid in 80 per cent. alcohol. Wash in alcohol, dehydrate, clear and mount.

Fieandt (*Arch. mikr. Anat.*, lxxvi, 1910—11, p. 125) fixes in Heidenhain's sublimate-trichloracetic mixture, and treats pieces for five to seven days with 96 per cent. alcohol, to be changed three times during the first twenty-four hours and daily in the following days. After dehydration the pieces are imbedded in paraffin as directed by Prantner, clearing with cedar oil and ligroin, and putting thence into a saturated solution of paraffin in ligroin in the incubator at 37° C. ; thence into paraffin of 52° C. melting point. The sections, 3 to 5 μ thick, are stuck to slides, freed from sublimate by the usual iodine treatment, and then stained for twelve to twenty-four hours with Mallory's phosphotungstic hæmatoxylin. Dry with filter paper, differentiate for a few hours in 10 per cent. iron perchloride in absolute alcohol, blot once more with filter paper, wash, dehydrate and mount.

Neuroglia fibres, cytoplasm of neuroglia cells, and glia granules

stained in various shades of blue and greyish-blue ; all other elements yellowish-grey or yellowish-brown.

RANKE (*Ztschr. ges. Neurol. u. Psych.*, vii, 1911, p. 355) uses for similar purposes either celloidin sections of fœtal tissues fixed in picric acid alcohol or sections made by freezing from formalin (pathological) material. In the first case the sections are stuck to slides by pressing with filter paper and then pouring on them methyl alcohol until all celloidin is dissolved. He next stains them for a few minutes with his acid eosin-thionin solution (see further on), washes with water, and re-stains them, with the help of gentle heat, with 5 : 1000 Giemsa's "*Methylenazur I*" ; quick differentiation with distilled water ; 96 per cent. alcohol, cajeput oil, xylol, balsam. In the case of pathological material the sections are first treated with 1 per cent. osmic acid in order to stain fatty products of degeneration, etc., then pressed on to slides and stained as above. To prepare the acid eosin-thionin mixture, mix and shake repeatedly 1000 c.c. of each 1 : 1000 watery solution of eosin W.G. and 1 : 1000 watery solution of thionin. Leave for forty-eight hours, pour out the fluid part, and wash the sediment into a paper filter with distilled water until the wash-water is only a little stained. Dry what remains in the filter, and dissolve it in methyl alcohol in the proportion of 0·3 to 0·5 per cent.

1091. Alzheimer's Methods (*Histol. u. Histopathol. Arb*, iii, 1910, p. 406) :—

(*a*) "ALZHEIMER-MALLORY" *Method.* Fix in gliabeize + 10 per cent. formalin, wash and cut frozen sections. Wash rapidly first with distilled water and then for two minutes with distilled water rendered very slightly acid with acetic acid. From this put directly into the stain which consists of 10 c.c. 10 per cent. phosphomolybdic acid, 1·75 grm. of hæmatoxylin, 5 grm. phenol crystals, and 200 c.c. distilled water (ripened for two months), wash, dehydrate, clear and mount in balsam. (Specially suitable for demonstration of cell bodies and certain granules.)

WEIL (*loc. cit.*) stains paraffin sections in the hæmatoxylin for half to one hour after treating them for five minutes with 5 per cent. potassium bichromate. He advises thorough washing with 50 per cent. alcohol before further dehydration.

(*b*) "ALZHEIMER-MANN" *Method.* Fix in gliabeize + 10 per cent. formol. Cut thin frozen sections and transfer first for ten minutes to distilled water to which a few drops of 10 per cent. phosphomolybdic acid are added, and then for several hours to a saturated watery solution of phosphomolybdic acid. Wash twice rapidly in distilled water and stain for one hour in Mann's methyl blue eosin solution (1 per cent. methyl blue 35 c.c., 1 per cent. watery eosin 35 c.c., distilled water 100 c.c.). Wash in distilled water till no more stain is given off, transfer for one to two minutes to 96 per cent. alcohol, then absolute alcohol, xylol-balsam. This method may be used on paraffin sections, fixing material in 25 per cent. formalin and subsequently mordanting in 5 per cent. potassium bichromate for fourteen days before im-bedding in paraffin.

(*c*) "'*Fuchsin-light green*" *Method.* Fix for twenty-four hours in formalin, and thence transfer direct to Flemming's solution for eight days. Wash twelve to twenty-four hours and imbed in paraffin of 58° C. melting-point. Cut very thin sections (2 to 3μ), dry, remove paraffin and wash with 96 per cent. alcohol. Thence place in paraffin oven at 58° C. for one hour in a saturated watery solution of acid fuchsin. Wash twice with water till no more stain is given off, and differentiate for ten to twenty seconds in a mixture of 30 parts of saturated alcoholic picric acid and 60 parts of distilled water. Wash twice thoroughly in water and stain for twenty to fifty minutes in a half-saturated watery solution of light green. Wash quickly, first in water and then in 96 per cent. alcohol, absolute alcohol, xylol, balsam. (This method does not differentiate the neuroglia fibres, but gives a very complete picture of the structure of the supporting tissues. It also brings out sharply "fuchsinophil" and lipoid granules in the neuroglia cells.) BERTRAND and HADJIOLOFF (*Rev. Neurol.*, ii, 1927, 34, p. 752), stain with Ziehl's carbol-fuchsin on a hot plate and after washing the sections in distilled water until no more stain is given off they then stain with a saturated aqueous solution of light green for seven to fifteen minutes on a hot plate.

1092. Other Methods for Granules, etc. ALZHEIMER (*loc. cit.*) also stains *fuchsinophil granules* by treating Flemming fixed paraffin sections (see above) first for one hour at 37° C. in a saturated watery solution of copper acetate, and after two washes in distilled water, staining for half an hour in 10 per cent. alcoholic hæmatoxylin, 10 c.c. distilled water, 87 c.c. saturated lithium carbonate solution 3 c.c. Wash rapidly in water, alcohol, xylol, balsam.

For *Reich's π granules* he stains frozen sections of formalin material in 1 per cent. toluidin blue for one hour, washes thoroughly in distilled water and differentiates, less completely than for Nissl granules, with alcohol.

METHODS FOR PROTOPLASMIC NEUROGLIA OLIGODENDROGLIA AND MICROGLIA

1093. RAMÓN Y CAJAL'S **Gold Chloride and Sublimate Method** (*Trab. Lab. Invest. Biol.*, Madrid, xi, 1913, pp. 219 and 255; xiv, 1916, p. 155). At first Cajal used to harden pieces of quite fresh tissues in 14 per cent. formalin, but in his successive papers he recommended fixing from two to ten days in —

Formol	15 c.c.
Ammonium bromide	1·5–2 grm.
Distilled water	85 c.c.

Relatively thick sections (20 to 25 μ) are made by the freezing

method, and collected in distilled water to which a few drops of formalin have been added. After a quick wash, batches of four to six sections are each transferred into glass dishes of about 6 cm. in diameter, and each containing 15 c.c. of a mixture of—

Distilled water	60 c.c.
Mercuric chloride	0·5 grm.
1 per cent. gold chloride (Merck, *brown variety*)	10 c.c.

After about four hours the sections will be found to have become an intense purple, and can be passed, for five to ten minutes, into a fixing bath consisting of—

Concentrated solution of sodium hyposulphite	5 c.c.
Distilled water	70 ,,
Alcohol	30 ,,
Concentrated solution of sodium bisulphite	5 ,,

Wash in 50 per cent. alcohol, lift sections on to slides, dry with filter paper, wash with absolute alcohol, clear with origanum oil, wash with xylol, and mount in balsam.

Best results are obtained by keeping the glass dishes, with the sections and the gold chloride-sublimate mixture, at a temperature of 18° to 20° C. If the reagent is freshly prepared, the reaction will be complete in about four to six hours. At temperatures between 14° and 17° C. three or four hours more are necessary to obtain good stains. With temperatures below 14° or 12° C. it is very difficult to obtain any reaction at all. One may have recourse to temperatures above 20° C., up to 27° or 30° C. in special cases, as Del Río-Hortega has done for the neuroglia of the pineal body. More diluted gold baths may be used for economical reasons, but in this case one must have recourse either to higher temperatures or to greater lengths of time. To proceed quicker, one may either double the proportion of sublimate in the formula given above or double the proportion of gold chloride and treble that of sublimate. A good means to obtain rapid and vigorous reactions consists in adding to the gold chloride-sublimate bath either 2 to 3 drops of a 1 : 100 solution of erythrosin or a minute quantity of the dry dye, enough to impart to the bath a slightly orange tone. All other conditions being the same, results are greatly influenced by the length of time during which the pieces have been kept in the fixing fluid. As a rule, they begin to be ripe for cutting from the end of the third day, and they continue to be in a state favourable for obtaining good reactions for another five or six up to fifteen or twenty days. Good stains may be exceptionally obtained after

two months of hardening. The capacity for taking the gold disappears first from the protoplasmic, and then from the fibrous, neuroglia.

We have found that this method can be successfully used on material fixed in formol saline for several weeks or even months. Frozen sections are cut at 20 μ and left in formol bromide at laboratory temperature overnight. After a quick wash they are then placed in the following solution, which must be freshly prepared :—

Mercuric chloride	0·5 to 1·0 grm.
One per cent. gold chloride (Merck's brown, or yellow crystals) . .	20 c.c.
Distilled water	30 ,,

The sections stay in this solution for half an hour or longer in the incubator at 37° C. and are then fixed as in the original Cajal method. This stronger solution will often be found preferable to Cajal's formula. (See also RAILEANN, *Rev. Neurol.*, i, 1930, p. 1018).

CORTEN (Wertham's "The Brain as an Organ," the Macmillan Company, New York, 1934) uses tissue fixed in formol saline. Frozen sections cut at 25 μ are placed in ammonium bromate, 15 c.c.; neutral formalin, 100 c.c.; distilled water, 400 c.c.; the solution being heated till it steams. Sections are then transferred to antiformin, 3 c.c.; distilled water, 2 c.c.; 96 per cent. alcohol, 8 c.c., for six to fifteen seconds. They must be moved about in this solution. After washing in two changes of distilled water sections are treated with Cajal's gold sublimate solution, the use of a higher concentration of mercuric chloride being recommended.

By means of Cajal's method two categories of neuroglia elements become stained a dark purple on a much lighter purplish background. The first category consists of *neuroglia cells* provided with a changing number of variously ramified protoplasmic processes, which inter-cross with those of other cells, and thus give origin to Cajal's *pleurigenic plexus*. These neuroglia cells prevail in the grey layers of the human cerebral cortex, and form the bulk of the *protoplasmic neuroglia* (§ 1082). In Cajal's preparations they appear beset with vacuoles, situated both within their cytoplasm and along their processes. The vacuoles or spaces are occupied by granules (*gliosomes*), which may be stained either by Cajal's uranium nitrate method (§ 723) (superficial sections) or by methods generally used for the demonstration of mitochondrial formations as well as by the methods of Eisath and Fieandt. The other category of neuroglia elements shown by the gold chloride and sublimate method consists of *astrocytes*, viz,. of neuroglia cells, also provided with a changing number of processes, but chiefly characterised by the *presence of fibres* which,

though a product of differentiation of the protoplasmic portions of the astrocytes, never become entirely independent of the latter. These fibres appear to correspond to those stainable by the methods described in §§ 1083 *et seq.* The astrocytes prevail in the white matter of the central nervous system, and form the bulk of the *fibrous neuroglia* (§ 1082).

The gold chloride and sublimate method leaves unstained *a third category of elements*, the existence of which was at first recognised by Cajal by means of this negative character, but they were subsequently studied by him in superficial sections of pieces stained by his uranium nitrate method and other cytological methods. The cells belonging to the category now considered appear in uranium nitrate preparations as roundish elements, but, as a matter of fact, they also are provided with a changing number of variously-ramified protoplasmic processes (see § 1082). As Cajal was not able to come to any definite conclusion in regard to their nature, he proposed to term them the "*third element,*" *i.e.*, a category of cells which though non-nervous in character, do not plainly form part either of the connective tissue (blood-vessels, pial septa) or of the neuroglia, this term being, in Cajal's opinion, reserved for those elements which are genetically derived from an evolution of the ependymal epithelium.

1094. ACHUCARRO'S **Tannin Method and** DEL RÍO-HORTEGA'S **Modifications.** The methods described in this paragraph can be considered as the direct outcome of various efforts at modifying the Bielschowsky method for sections (§ 1018) in such a way as to obtain a neuroglia stain. As a matter of fact, they all stain both neuroglia cells (astrocytes) and connective tissue elements. In other words, they are not elective, and may be used for the study of reticular tissue in non-nervous organs, as well as of other histological details in nervous and non-nervous tissues.

PERUSINI'S *modification of* BIELSCHOWSKY'S *method* (*Neurol. Centrbl.*, xxix, 1910, p. 1256) should be first remembered. Pieces of fresh material were fixed in Weigert's formalin-copper acetate-chromium fluoride mixture for neuroglia stain (§ 1083), cut by the freezing method, and stained as by Bielschowsky's method for sections, without pyridine treatment. Achúcarro did the same, except for silvering by Ramón y Cajal's reduced silver process.

ACHUCARRO'S *tannin method* (*Bol. Soc. Espan. Biol.*, Madrid, 1911, p. 139) consisted in putting sections made from frozen formol material into a cold-saturated solution of tannin and warming this until vapour arose. Without waiting for the tannin to become cool again, the sections were, one by one, quickly rinsed in water and put to stain for about ten minutes into three successive glass dishes, each containing 10 c.c. of distilled water and 6 to 8 drops of Bielschowsky's ammoniacal silver nitrate-and-oxide bath, prepared beforehand, as described in § 1018. As soon as they turned dark yellow, they were transferred into

10 per cent. formalin, and, after about ten minutes, washed, dehydrated and mounted.

The results obtained by such a method were rather uncertain, and Achúcarro himself felt the necessity of modifying it in the following way, published by Del Río-Hortega (*Trab. Lab. Invest. Biol.*, Madrid, xiv, 1916, p. 181):—(1) Fix pieces, 2 to 3 mm. thick, for two or three days in formalin neutralised with ammonia. (2) Make sections of 10 μ, and mordant them in 10 per cent. tannin until vapour arises. (3) Without waiting for the tannin to become cool, wash the sections in distilled water alkalised with a few drops of ammonia until they have again acquired their flexibility. (4) Treat them with the diluted ammoniacal silver nitrate solution as described above, but adding only 2 or 3 drops of it to every 10 c.c. of distilled water. (5) Reduce in 20 per cent. formalin, either neutralised as for fixing, or (according to Del Río-Hortega) containing an excess of ammonia, say, 6 to 8 drops to every 10 c.c. of 20 per cent. formalin.

Del Río-Hortega (*op. cit.*) found that the method could be further modified, and usefully employed for the staining not only of the neuroglia, but also of *centrosomes* of nerve-cells and neuroglia cells, *mitochondria*, *secretion granules*, *intra-epithelial fibrils*, *reticular tissue*, *collagenous fibres*, etc. The modifications proposed by Del Río-Hortega for these various purposes are *four* in number, and known as the *variants of Achúcarro's method.*

Modification I. Suitable for the staining of fibrous neuroglia as well as for elastic membranes and connective tissue cells. (1) Fix tissues for no less than ten days in 10 per cent. formalin. (2) Make sections by the freezing method, and mordant them for five minutes in 3 per cent. tannin kept at a temperature of 50° to 55° C. (3) Wash them in distilled water alkalised with ammonia, and transfer them successively into three glass dishes, each containing 1 c.c. of ammoniacal silver nitrate, prepared as described in § 1022, and 10 c.c. of distilled water. (4) As soon as they have taken a distinct yellowish-brown colour, wash them in distilled water and reduce them in a 1 : 500 gold chloride solution kept for twenty or thirty minutes at a temperature of about 40° to 45° C. (5) Fix with 5 per cent. sodium hyposulphite, wash, dehydrate and mount as usual.

Modification II. Good chiefly for reticular tissue and its histogenesis. Material may be fixed either in 10 per cent. formalin or Bouin's fluid, or alcohol; if one or the other of these last two fluids has been used, it is advisable to re-transfer pieces for a few days into a formalin solution. Sections should, as a rule, be made by the freezing method, but pieces may also be imbedded in celloidin, this being dissolved after cutting. The sections, however obtained, are mordanted for five minutes at 50° to 55° C. or for fifteen to thirty minutes at 40° to 45° C. in a 1 per cent. *alcoholic* solution of tannin. Stain as in Modification I;

reduce for half a minute in 20 per cent. formalin, neutralised by shaking with chalk, wash, dehydrate and mount.

Modification III.—Particularly good for collagenous fibres, but also for neuroglia fibres. Proceed as in Modification II until the sections are placed in the staining bath; keep them therein until brown; reduce and fix as in Modification I.

Modification IV (*op. cit.*, xvi, 1918, p. 375, *note*). Suitable for the demonstration of the protoplasmic neuroglia. Frozen sections of formalin material are treated for some minutes at 45° to 50° C. with a mixture of tannin, 3 grm.; ammonium bromide, 1 grm.; distilled water, 100 c.c. Wash and stain as in Modification I; reduce in 20 per cent. formalin neutralised with chalk; tone with 0·2 per cent. gold chloride; fix, wash, dehydrate and mount as usual.

Ramón y Cajal (*Trab. Lab. Invest. Biol.*, xviii, 1920, p. 129) stains *neuroglia astrocytes* by a modified Bielschowsky technique, fixing in formol-bromide solution as above and after making frozen sections refixing them in 6 per cent. ammonium bromide in either water or 12 per cent. neutral formalin for four to six hours in the oven at 37° C. or for eight to twelve hours at room temperature. Wash quickly in two changes of water and place in a silver bath made in the following way. Add 12 drops of 40 per cent. sodium hydroxide to 10 c.c. of 10 per cent. silver nitrate, wash precipitate six to seven times with distilled water and dilute with 60 to 70 c.c. of distilled water before dissolving with ammonia. Of this strong solution put 3 to 5 c.c. into each of several dishes and add to each 15 c.c. of distilled water and 2 to 4 drops of pure pyridin. Put sections into these dishes and keep them first for five to ten minutes in the cold and thereafter warm till the sections take a tobacco colour. Keeping the sections warm, wash for a few seconds in a large amount of distilled water and place in 20 per cent. neutral formol. Wash rapidly in water and tone in Merck's brown gold chloride for several hours in the cold or for ten to twenty-five minutes at 37° C. Fix, dehydrate, mount.

He also (*Arch. Suisses de Neur. & Psych.*, xiii, 1923, p. 187) recommends his uranium nitrate method, as devised for staining Golgi's internal apparatus (§ 724), for the demonstration of protoplasmic and fibrous neuroglia cells and also for gliosomes and lipoid inclusions.

1095. Del Río-Hortega's **Carbonate of Silver Method** (*Trab. Lab. Invest. Biol.*, Madrid, xv, 1917, p. 367, and xviii, 1920, p. 37; *Bol. Soc. Esp. Biol.*, viii, 1918). Pieces of quite fresh nervous tissues are fixed in Cajal's ammonium bromide-formalin mixture, and kept therein for different periods of time, according to the purposes in view. If it is desired to stain the *protoplasmic neuroglia*, pieces are best fixed for twenty to thirty or forty days;

after this time they are for some months in a condition particularly suitable for the staining of the *fibrous neuroglia.* But if the time of fixation is limited to *one* or *two* days at the temperature of about 35° C., or to *two* up to *four* days at room temperature, the tissues are in a state favourable to the impregnation of Cajal's "*third element*" (§ 1093), which Del Río-Hortega proposes to term either *microglia* or *mesoglia,* the first of these two denominations being simply used with reference to the smallness of the elements thus named, the second implying that they do not belong to the neuroglia as this term is understood by Cajal and his pupils. For the staining one may choose one or the other of the following three processes :—

Process I, for protoplasmic and fibrous neuroglia. Sections made by the freezing method are washed in two or three changes of distilled water and transferred into a crystallising basin containing 5 or 10 c.c. of ammoniacal silver carbonate solution, prepared as follows :—To 50 c.c. of 10 per cent. silver nitrate an equal or greater quantity of cold-saturated lithium carbonate solution is added, so as to precipitate all silver in the form of silver carbonate. The fluid part is poured off, and the precipitate first washed with 200 to 300 c.c. of distilled water, and then taken up with about 50 c.c. of diluted ammonia, by means of which it is entirely dissolved. The solution is diluted with distilled water up to a total volume of 250 c.c. and poured into a dark brown bottle, where it keeps indefinitely, if put away in some dark place.

The crystallising basin, with the ammoniacal silver carbonate and the sections placed therein, is warmed, either in an incubating stove at 45° to 50° C. or over a flame, until the sections become a greyish-yellow colour. This requires only a few minutes if the sections are moved about so that they may stain uniformly. Good results may be also obtained by staining at 35° C. for twelve to fourteen hours or at room temperature for one or two days. Without waiting for the silver solution to become cool, the sections are quickly washed in distilled water and then transferred, one by one, into 20 per cent. formalin neutralised with chalk. After one or two minutes, the reduction is complete, and the sections may be washed, toned, fixed and dehydrated, cleared with a mixture of carbolic acid 5 parts, xylol 45 parts, creosote 50 parts, and mounted in balsam.

Process II, for microglia. Sections are made as above, and then treated for ten or fifteen minutes at 50° or 55° C. with the bromide-formalin solution used for fixing. After washing in two or three changes of water, one continues as in Process I, but warming the ammoniacal silver carbonate solution at 50° or 55° C. until the sections are *dark* yellow.

Process III, also for *microglia.* The *pieces* are warmed for

ten minutes in the fluid used for fixing, and then cut by the freezing method. The sections are washed in distilled water and stained for ten to thirty minutes, either at room temperature or by careful gentle warming, with an ammoniacal silver carbonate solution, prepared by adding to 10 c.c. or 10 per cent. silver nitrate, first, 30 c.c. of 5 per cent. sodium carbonate, then ammonia, drop by drop, until the precipitated silver carbonate is dissolved, and, lastly, distilled water up to a total volume of 150 c.c. The sections are kept in the impregnating bath for from ten to thirty minutes at room temperature, but they should nevertheless remain almost colourless.

They are then transferred directly without washing to 1 per cent. formalin and kept moving about in this, either by blowing on the surface or by moving them with a glass-rod until reduction is complete. Finish as in Process I.

The strength of the ammoniated silver carbonate solution used in Process III may be varied. Hortega has more recently used 5 c.c. of 10 per cent. silver nitrate and 20 c.c. of 5 per cent. sodium carbonate, and after dissolving in ammonia made up with distilled water to 45 c.c. (strong solution) or 100 c.c. (weak solution). The strength of formalin may also be varied up to 10 per cent.

The above refers to material fixed in Cajal's ammonium bromide-formalin mixture; if nervous tissues are fixed in 10 per cent. formalin and sections treated as in Process I, nerve-cells and axis-cylinders become stained as by Bielschowsky's method. If formol sections of non-nervous tissues are treated in the same way, the reticular tissue becomes stained.

1096. Other Silver Methods for Neuroglial Astrocytes. GLOBUS (*Arch. Neurol. and Psychiat.*, xviii, 1927, p. 263) recommends the following procedure for formalin fixed material before impregnation by Cajal's method for neuroglia or Hortega's third process for microglia (see § 1095). Frozen sections are thoroughly washed in distilled water, placed in 10 per cent. ammonia for twenty-four hours, washed again in distilled water, mordanted for two to four hours in 10 per cent. hydrobromic acid and after a final wash in weak ammonia water are stained by the selected technique.

LUGARO (*Arch. Suisse de Neurol. et de Psychiat.*, xxix, 1932, p. 282) uses 15 per cent. formalin for fixing pieces of tissue 3 to 4 mm. thick before staining the protoplasmic neuroglia with varying proportions of a mixture of silver bromide and silver iodide. The pieces are stained in bulk for five to six days, allowing 10 c.c. of solution for each piece, and after three hours in the solution 7 c.c. of 25 per cent. formalin are put in for each piece of tissue. The stock solutions are: A, 9 per cent. sodium hyposulphite; B, 2 per cent. silver bromide in 9 per cent. sodium hyposulphite; C, 0·3 per cent. silver iodide in 9 per cent. hyposulphite. These

can be used in the proportions of 4, 5, 6, 7, 8, 9, 10 and 11 parts of (A) to 16, 15, 14, 13, 12, 11, 10 and 9 parts of (B), and 1, 2, 4, 5, 6, 7, 8 and 12 parts of (C). After staining, the tissue is rinsed in running water and frozen sections are cut at 35 μ.

Bolsi (*Riv. d. Pat. Nerv. e Ment.*, xxxii, 1927, pp. 51 and 898) fixes tissues in 5 c.c. each of pyridine and acetone; formol, 15 c.c.; ammonium bromide, 3 grm.; distilled water, 75 c.c., for six days to six months. Frozen sections are cut at 15 μ and well washed in distilled water before being stained in an ammoniacal silver solution prepared by adding ammonia to 10 c.c. of 2 per cent. silver nitrate until the resultant precipitate is dissolved and then adding 20 c.c. of glycerin. The sections are reduced in 20 per cent. formalin. This method stains neuroglia and microglia. To stain microglia only, frozen sections are passed from distilled water into glycerin, 40 c.c.; ammonia, 100 drops; distilled water, 160 c.c.; being constantly moved about in this solution for five minutes before being stained in 2 per cent. silver nitrate for one minute and reduced in 1 to 2 per cent. formalin for five minutes.

Dubrauszky (*Zeitschr. f. d. ges. Neurol. u. Psychiat.*, cxxvi. 1930, p. 230) fixes small pieces of brain for forty-eight hours in formalin, 6 c.c.; sodium bicarbonate, 6 grm.; 15 to 20 per cent. ammonia, 16 drops; distilled water, 100 c.c. Frozen sections are washed first in weak ammonia water and then in distilled water and immersed in a silver bath prepared by adding 1 drop of strong ammonia to 1 c.c. of 20 per cent. silver nitrate, diluting with distilled water to 15 c.c., and filtering the solution before use. The time of impregnation is about fifteen seconds. The sections are reduced in ½ per cent. formalin to which 1 drop of silver nitrate for each 20 c.c. of formalin is added.

Vizioli (*Riv. d. Neurol.*, v, 1932, p. 165) places frozen sections of formalin fixed material in Dubrauszky's fixative (see above), in which the sodium bicarbonate is replaced by sodium carbonate. He then transfers them to Bolsi's ammoniacal glycerin solution (see above) and after a rapid wash, to Bolsi's ammoniacal silver nitrate. Very dilute formalin (30 to 40 parts in 1000 c.c. of water) is used for reduction, which takes about one to two hours.

1097. Other Methods for Microglia. Penfield (*Am. Journ. Path.*, iv, 1928, p. 153) places frozen sections of formalin fixed material in weak ammonia water overnight and then transfers them directly to 5 per cent. hydrobromic acid for one hour at 37° C. After being washed in three changes of distilled water, sections are put into 5 per cent. sodium carbonate for one to six hours, and are subsequently stained in Hortega's silver carbonate solution diluted to 75 c.c., until they turn a light brown colour.

Kanzler (*Zeitschr. f. d. ges. Neurol. u. Psychiat.*, cxxii, 1929, p. 416) heats frozen sections of formalin fixed tissues till vapour

rises in ammonium bromide, 15 grm.; 40 per cent. formaldehyde, 100 c.c.; distilled water, 400 c.c. Without washing, the sections are placed in antiformin, 3 c.c.; 95 per cent. alcohol, 8 c.c.; distilled water, 2 c.c.; for five to eight seconds. After a thorough washing in two changes of distilled water the sections are transferred to an ammoniacal silver solution prepared by mixing 5 c.c. of 10 per cent. silver nitrate with 15 c.c. of 10 per cent. caustic soda and dissolving the precipitate with ammonia. The sections must be kept moving in this solution for eight to ten seconds. They are reduced in 2 per cent. formalin, washed and toned.

Belezsky (*Virchow's Arch. f. Path. Anat.*, cclxxxii, 1931, p. 214) has stained microglia in celloidin sections. After a prolonged wash in distilled water the sections are placed for twenty minutes in the following solution: to 5 c.c. of 17 per cent. silver nitrate add 5 drops of 40 per cent. caustic soda. Dissolve the precipitate with 25 per cent. ammonia and dilute about forty times. The sections are reduced in 10 per cent. formalin after a quick wash in distilled water, the reduction being accelerated by heat.

Stern (*Zeitschr. f. d. ges. Neurol. u. Psychiat.*, cxxxviii, 1932, p. 769) also stains microglia in celloidin sections, which, after prolonged washing in distilled water are placed in the following solution for five to thirty seconds: to 4 c.c. of 10 per cent. silver nitrate add 2 or 3 drops of strong ammonia and then a few more drops to dissolve the precipitate which forms. Dilute with 20 c.c. of distilled water. Transfer sections without washing to 10 to 20 per cent. formalin, keeping the sections moving until reduced. If the sections are left longer (thirty to sixty seconds) in the ammoniacal silver or are allowed to sink to the bottom of the reducing dish, oligodendroglia will be stained.

Weil and Davenport (*Arch. Neurol. and Psychiat.*, xxx, 1933, p. 175) have modified the above method for staining microglia in celloidin sections. They add 10 per cent. silver nitrate drop by drop from a burette to 2 c.c. of concentrated ammonia, shaking the solution to avoid a precipitate. The end point is given by a slight opalescence. Sections are left in this solution for ten to twenty seconds and reduced without washing in a solution of formalin, 1 part; distilled water, 5 parts; moving the sections about until they are of a coffee-brown colour.

Del Río Hortega's Method for Pineal Parenchyma. (Horrax and Bailey, *Arch. Neurol. and Psychiat.*, xiii, 1925, p. 423.) (Originally published in *Arch. de Neurobiol.*, iii, 1922, p. 351. (1) Fix in 10 per cent. formalin for at least two days. (2) Cut thin frozen sections. (3) Place for twenty-four hours at room temperature in: 10 per cent. silver nitrate, 10 c.c.; pure pyridine, 3 drops; the sections become dark yellow. (4) Wash in distilled water to which 2 drops of pure pyridine have been added.

(5) Colour in the following solution, heating to about 50° C.: solution of silver carbonate, 10 c.c.; pure pyridine, 3 drops; the sections become a dark sepia colour. (6) Wash in distilled water. (7) Reduce in 10 per cent. formalin. (8) Tone with gold chloride, warming the sections slightly to intensify the colour (9) Fix, wash, dehydrate, clear and mount.

1098. Oligodendroglia. ROBERTSON (*Scot. Med. & Surg. Journ.*, Jan. 1899, and "Text-book of Pathology in Nervous Diseases," 1900) stained oligodendroglia by placing thin pieces not more than $\frac{1}{16}$ of an inch in thickness in a mixture of equal parts of $\frac{1}{2}$ per cent. platinum bichloride (? hydrochloro-platinic acid) and 20 per cent. formalin and leaving them in a dark place for several weeks until they are blackened all over. Thereafter he cut frozen sections and mounted them without further staining.

PENFIELD (*Brain*, xlvii, 1924, p. 430) obtained good staining of oligodendroglia by fixing in ammonium bromide-formalin solution for two to twelve hours only, followed by thirty-six to forty-eight hours in 95 per cent. alcohol, and after a thorough washing of the blocks proceeding as in Hortega's Process III. (§ 1095). He has also (*Amer. Journ. Path.*, vi, 1930, p. 45) stained oligodendroglia in material fixed in formalin by placing blocks of tissue in 15 per cent. ammonia for twenty-four hours washing in running water overnight and then transferring them to formalin, 20 c.c.; urea, 4 grm.; potassium iodide, 6 grm.; distilled water, 80 c.c., for a week, thereafter cutting frozen sections, placing them in 4 per cent. urea overnight and staining in strong, undiluted silver carbonate solution (10 per cent. silver nitrate, 5 c.c.; sodium carbonate, 20 c.c.; ammonia to dissolve precipitate), for one minute to one and a half hours. The sections are washed rapidly in 60 per cent. alcohol and reduced in 1 per cent. formalin.

CONE (*Journ. f. Psych. u. Neur.*, xxxiv, 1926, p. 204) stains neuroglia astrocytes and oligodendroglia in tissue fixed in Weigert's "gliabeize" for seven days (with 10 per cent. formalin on first day only), by placing frozen sections, after washing, in 10 per cent. phosphomolybdic acid for twenty-four hours, thereafter staining in weak silver carbonate (see § 1095) for one to two minutes (for oligodendroglia), or for five to ten minutes (for astrocytes) and transferring directly to 1 per cent. formalin.

HORTEGA (*Mem. d. l. Real. Soc. Esp. d. Hist. Nat.*, 1928) fixes pieces of tissue not more than 2 to 3 mm. thick in potassium bichromate, 3 grm.; chloral hydrate, 2 to 5 grm.; 10 per cent. formol, 50 c.c., for two to three days. Dial or veronal may be used instead of chloral. After a rapid wash in distilled water the pieces are put into 1·5 per cent. silver nitrate for two to three days in the dark. Frozen sections are made and as impregnation is never very deep it is advisable to mount the first sections cut.

BAILEY and BUCY (*Journ. Path. and Bact.*, xxxii, 1929, p. 735) stain oligodendroglia by Penfield's method for microglia (§ 1097),

using 10 per cent. ammonia instead of weak ammonia for the preliminary washing overnight and substituting a saturated solution of lithium carbonate for sodium carbonate in the silver carbonate solution.

WEIL and DAVENPORT (*Arch. Neurol. and Psychiat.*, xxx, 1933, p. 175) stain oligodendroglia in celloidin sections by treating the sections with 10 per cent. ammonia water before staining them in a solution prepared as for staining microglia in celloidin sections (see § 1097), but using 15 per cent. silver nitrate. Weaker formalin (10 per cent.) is used for reduction and the sections are allowed to drop to the bottom of the dish until the celloidin is stained brown. They are then moved about until the sections are coffee colour.

1099. For **Mitochondria and Gliosomes,** DEL RÍO HORTEGA (*Bol. de la Soc. Esp. de Hist. Nat.*, 1925, p. 34) fixes small pieces for two to three days at 25° to 35° C. or for four to eight days at room temperature in a mixture of 10 per cent. formalin to which is added either 6 to 8 per cent. of iron alum or 1 to 2 per cent. uranium nitrate. He uses three modifications in staining these. (*a*) Frozen sections are made, washed in distilled water and stained in the silver carbonate solution used in Process III, and thence reduced in ½ per cent. formalin, after washing for mitochondria, but without washing for gliosomes. Tone in gold and fix in hyposulphite. (*b*) Specially for *gliosomes*, but revealing also the processes of neuroglia cells. Fixation for two to eight days in iron-alum formalin, or after fixation in formol-bromide place for twenty-four hours in iron-alum solution. Frozen sections are washed first in two or three changes of water containing a few drops of ammonia and then in pure distilled water. Stain in 10 c.c. of silver carbonate solution to which is added 3 drops of pyridin, heating to 45° to 50° C. until the sections take a light tobacco colour. Wash one to three minutes in distilled water and reduce in 10 per cent. formalin. (*c*) Specially for the specific granules of ependymal cells. Fix in iron-alum and formol-bromide solution (10 per cent. formol, 2 per cent. ammonium bromide, 6 to 8 per cent. iron alum) for three to four days. Frozen sections are washed as in (*b*) and transferred first to 2 per cent. silver nitrate for ten to fifteen minutes, then after a rapid wash to silver carbonate for one minute. After the most rapid wash possible they are reduced in 1 per cent. formalin.

Methods (*a*) and (*c*) after formol-uranium nitrate fixation are specially suitable for *gliosomes* and for *interfascicular oligodendroglia*.

METHODS FOR SENILE PLAQUES

1100. MARINESCO (*L'Encephale*, xxiii, 1928, 697) places pieces of formalin fixed material not more than 8 mm. thick in 96 per

cent. alcohol for twelve to twenty-four hours, washes them in distilled water and then puts them into 1·5 per cent. silver nitrate to which 10 per cent. pyridine has been added for twenty-four to forty-eight hours at 37° C. After a rapid wash in distilled water reduction is effected by a solution of 2 per cent. pyrogallic acid, 90 c.c., pyridine 10 c.c., in ten to twelve hours. Frozen sections may be cut or the material may be imbedded in paraffin.

BRAUNMÜHL (*Zeitschr. f. d. ges. Neurol. u. Psychiat.*, cxxii, 1929, p. 317) uses frozen sections of well-fixed formalin material, which after a thorough wash in distilled water are transferred to 20 per cent. silver nitrate for half an hour at 50 to 60° C. The sections are washed in 1 per cent. ammonia and reduced in 20 per cent. neutral formalin in tap-water for one to three seconds. The washing in ammonia is repeated and the sections are then left in formalin for five minutes, subsequently being well washed in distilled water. They may be toned in gold chloride if desired.

DIVRY (*Riv. d. Pat. Nerv.*, xl, 1932, p. 489) heats thin frozen sections of formalin fixed material until vapour rises in Hortega's solution for microglia 5 c.c., distilled water 5 c.c., pyridine 10 drops. After a quick wash in distilled water the sections are reduced in 10 per cent. neutral formalin, washed well in distilled water, and mounted in gelatin-glycerin.

Although the above methods are more rapid, we have found that Da Fano's modification of Bielschowsky's method for neurofibrils is equally suitable for the demonstration of senile plaques.

RETINA *

1101. Fixation and Hardening. Notwithstanding the *Encycl. mik. Technik.*, 2nd ed., p. 75, we hold that osmic acid is by far the best fixing agent. The retina of small eyes is best prepared by fixing the entire unopened bulb with osmium vapours.

Besides the sources quoted in the text, see SELIGMANN, *Die mikroskopischen Untersuchungsmethoden des Auges*, Berlin, S. Karger (Karlstrasse 13), 1899; GREEF, *Anleitung zur Mikr. Untersuch. d. Auges*, Berlin, Hirschwald, 3rd ed., 1910; the Art. "*Retina*" in *Encycl. mik. Technik.*, 2nd ed., p. 575; and D'AUTREVAUX, *Technique Histo-bacteriologique oculaire*, Paris, 1926.

SZENT-GYÖRGI (*Zeit. f. wiss. Mikr.*, xxxi, 1914), uses the following fluid :—

Acetone	125 c.c.
Glacial acetic	5 ,,
Formalin	40 ,,
Sublimate	4 grm.
Aq. dest.	100 c.c.

Leave whole small eyes in 100 c.c. of this mixture for two to three days, larger whole eyes six to seven days, after which one adds an additional 50 c.c. of acetone to the fixative and leaves for a further two or

* A. B. L. and J. G .G.

three days. Transfer to pure acetone for three or four days, renewing on the last day; then bring the eyes into a vessel of acetone, with a thick layer of desiccated calcium chloride at its bottom, for three or four days, renewing the $CaCl_2$, if necessary. Transfer from the acetone into a mixture of half ether, half absolute alcohol, then proceed as for celloidin imbedding.

According to Ranvier (*Traité*, p. 954) you may fix the eye of a triton (without having previously opened the bulb—the sclerotic being very thin) by exposing it for ten minutes to vapour of osmium. Then divide it by an equatorial incision, and put the posterior pole for a few hours into one-third alcohol.

Somewhat larger eyes, such as those of the sheep and calf, may be fixed in solutions without being opened. But it is generally the better practice to make an equatorial incision, and free the posterior hemisphere before putting it into the liquid.

The older practice was to use strong solutions of pure osmic acid alone; but most of the best recent work has been done with chromic mixtures following the osmium.

Dr. Lindsay Johnson tells me that he now gets the best results by suspending the globe over the steam of a 1 per cent. osmic acid solution raised to the temperature at which vapour is seen to be given off (but not to boiling point) for five minutes in the case of human adults, or for one to three minutes in the case of human infants, all monkeys and small mammals, as in them the sclerotics are very thin. As soon as the sclerotic is felt to be firm to the touch, it should be opened by a small nick with a razor just behind the ciliary body; or if the eye be that of an adult, the cornea and lens may be removed. The eye is then put for twelve hours into the mixture, § 50; it is then washed in running water, and suspended in a large volume of 2·5 per cent. bichromate of potash for two days, then passed gradually through successive alcohols, beginning with 20 per cent., and ending with absolute, taking five days from first to last.

Similarly Rochon-Duvigneaud (*Arch. Anat. Micr.*, ix, 1907, p. 317).

Other hardening liquids, however, also give good results, provided that the fixation by the osmic acid has been properly performed: amongst them *liquid of Flemming*, and that of Müller. Formaldehyde mixtures he does *not* recommend.

Leber (*Munch. med. Wochenschr.*, xli, 1894, p. 605; *Zeit. wiss. Mik.*, xii, 1895, p. 256) advises a solution of formol 1, water 10. After a few days' hardening in this, the eyes may be cut through, it is said, without derangement of the parts. The retina lies flat, and is at least as well preserved as with solution of Müller.

See also Heppel (*Arch. f. Ophthalm.*, xlv, 1898, p. 286; *Zeit. wiss. Mik.*, xvi, 1899, p. 79), who finds that formol fixes the lens badly, the retina well, so far at least as the absence of folds from shrinkage is concerned; and Herzog (*Arch. mik. Anat.*, lx, 1902, p. 517, and *Encycl.*

mik. Technik., p. 75), who also approves of formol, but insists that it should be *acid*, and adds 3 to 5 per cent. of acetic acid.

KOLMER (*Arch. Gesammte Phys.*, cxxix, 1909, p. 35), fixes for twelve to twenty-four hours in a mixture of 4 parts saturated solution of bichromate, 4 of formol of 10 per cent., and 1 of acetic acid.

BENDA (*Verh. Ges. Naturf. Ærzte*, lxxi, *Vers.*, 1900, p. 459) fixes in nitric acid of 10 per cent., and hardens in liquid of Müller, twenty-four hours in each.

ZÜRN (*Arch. Anat. Phys.*, *Anat. Abth.*, 1902, Supp., p. 106) advises (for mammals) fixing in saturated solution of sublimate in salt solution of 0·6 per cent., with 1 to 1½ per cent. of acetic acid after removing the anterior pole and the vitreous. Wash out in alcohol of 35 per cent. made 5 per cent. stronger each day up to 50 per cent.; then pass on to stronger and cedar oil and paraffin.

Zenker's and Bouin's fluids fix the retina excellently. It is usually best first to fix the eye entire, either in one of these solutions or in 10 per cent. formol, and after twenty-four hours to open it by a mesial incision after freezing it thoroughly in an ice and salt mixture. When formalin is used the eye should be returned to the fixative for a further twenty-four hours or more. After Zenker's or Bouin's fluid it may be washed at once to clear away the vitreous.

GREENFIELD and NEVIN (*Trans. Ophthal. Soc.*, liii, 1933, p. 170). recommend for human eyes injecting 1 c.c. of 20 per cent. formalin in saline into the vitreous with a fine hypodermic needle. After six to eight hours the sclerotic may be incised and the eye further fixed in 10 per cent. formalin or Zenker's fluid.

1102. Staining. For general views we recommend iron-hæmatoxylin, followed by säurefuchsin or picro-säurefuchsin, or preceded by Bordeaux; or Kernschwarz, followed by safranin, or the Ehrlich-Biondi stain.

The **Methylen blue** intra-vitam stain has given valuable results; see the methods of DOGIEL.

But the most important method is the **bichromate and silver** impregnation of GOLGI, first applied to this object by TARTUFERI (*Intern. Monatsschr.*, iv, 1887, p. 421). This author employed the rapid process. So also RAMÓN Y CAJAL (*La Cellule*, ix, 1893, p. 121) with the double-impregnation process, § 1028. To avoid the formation of precipitates on the tissues, he covers the retina, before silvering, with a piece of peritoneal membrane, or a thin layer of collodion. Or, better, he *rolls* the retina (*op. cit.*, p. 130). After removing the vitreous, the retina is cut away around the papilla with a punch or fine scalpel, and separated from the choroid. It is then rolled up (after being cut into quadrants or not), so as to form a solid block. This is painted with 2 per cent. celloidin, which is allowed to dry for a few seconds, and the whole is put into the bichromate mixture, and further treated as a solid mass of tissue.

RAMÓN Y CAJAL also employs his neurofibril silver method, see

Intern. Monatsschr. Anat. Phys., xxi, 1905, p. 393, and *Trab. Lab. Inv. Biol.*, xv, 1920, p. 1.

Golgi's **sublimate impregnation** (Cox's form) has also been successfully employed by Krause and Ramón y Cajal.

The bichromate and silver method serves for the study of the fibres of Müller and neuroglia cells, as well as neurones. Weigert's neuroglia stain does not give good results.

After Zenker fixation Mallory's phosphotungstic acid hæmatoxylin may be used.

Lennox (*Arch. f. Ophthalm.*, xxxii, 1886, 1; *Zeit. wiss. Mik.*, iii, 1886 p. 408) has used Weigert's hæmatoxylin method.

Kuhnt (*Jen. Zeit. Naturw.*, Bd. xxiv, 1890, p. 177) employs Pal's modification. Similarly Schaffer (*Sitzb. Akad. wiss. Wien.*, xcix, 1890, *Abth.* 3, p. 110; *Zeit. wiss. Mik.*, viii, 1891, p. 227). These methods give a differential stain of rods and cones.

For the zonula and ciliary body see Mawas, *Arch. d'Anat. micr.*, xii, 1910, p. 103.

1103. Dissociation. For maceration preparations you may use weak solutions (0·2 to 0·5 per cent.) of osmic acid for fixation and then macerate in 0·02 per cent. chromic acid (M. Schultze), or in iodised serum (M. Schultze), or in dilute alcohol (Landolt), or in Müller's solution, or (Ranvier, *Traité*, p. 957) in pure water, for two or three days. Thin (*Journ. of Anat.*, xiii, 1879, p. 139) obtained very good results by fixing for thirty-six to forty-eight hours in one-third alcohol, or in 25 per cent. alcohol, and then staining and teasing.

Schiefferdecker macerates fresh retina for several days in the methyl mixture, § 577.

Krause (*Intern. Monatsschr. Anat.*, i, 1884, p. 225) recommends treatment for several days with 10 per cent. chloral hydrate solution; the rods and cones are well preserved.

INNER EAR

1104. Inner Ear, Dissection. For the dissection of the human ear see Politzer, "Die anatomische u. histologische Zergliederung d. menschlichen Gehöroganes," Stuttgart (Enke), 1889 (*Zeit. wiss. Mik.*, vii 1890, p. 364). Amongst the lower mammalia, the *guinea-pig* is a favourable subject, as here (as with some other rodents) the cochlea projects freely into the cavity of the bulla, and may be easily removed with a scalpel and brought into a fixing liquid, and opened therein. With fishes and amphibia also the membranous labyrinth may easily be got away.

1105. Preparation. Schwalbe (*Beitr. z. Phys.* (C. Ludwig's Festschr.), 1887, p. 200). Fix (cochlea of guinea-pig) for eight to ten hours in "Flemming," wash in water, decalcify (twenty-four hours is enough) in 1 per cent. hydrochloric acid, wash the acid out, dehydrate, and imbed in paraffin.

Prenant (*Intern. Monatsschr. Anat.*, ix, 1892, p. 28). Open the cochlea in solution of Flemming or of Hermann, and fix therein for four to five hours. Avoid decalcification as far as possible, but if necessary take 1 per cent. palladium chloride. Make paraffin sections.

Isolation preparations of the stria vascularis may be made by putting a cochlea for a day into 1 per cent. solution of osmic acid, then for four to five days into 0·1 per cent. solution; the stria may then be got away whole.

Katz (*Zeit. wiss. Mik.*, xxv, 1908, p. 111) fixes the inner ear, opened, for one or two hours in 30 c.c. of 0·5 per cent. osmic acid with 5 drops of acetic acid, then adds 10 drops of acetic acid and 60 c.c. of chromic acid (or platinum chloride) of 0·5 per cent. and leaves it for four days therein. He then rinses, puts for twelve to twenty-four hours into pyroligneous acid or pyrogallol or tannin solution, decalcifies (not necessary for mice) in 200 parts of water with 1 of chromic acid and 4 to 10 of nitric or hydrochloric acid, and imbeds in celloidin or sometimes paraffin.

Similarly Wittmaack, see § 1071.

Bielschowsky and Bruehl (*Arch. mik. Anat.*, lxxi, 1908, p. 27) fix the petrous in formol of 20 per cent., decalcify it in nitric acid of 5 per cent., wash this out, and put back for a few days into the formol, cut by the freezing method and silver by the neurofibril method (§ 1021—twenty-four hours in nitrate of 4 per cent., but only a few minutes in the oxide bath).

Similarly Mullenix (*Bull. Mus. Comp. Zool. Harvard Coll.*, liii, 1909, p. 215).

Stein (*Anat. Anz.*, xvii, 1900, p. 398) decalcifies in celloidin by the method of Rousseau. So also Kishi (*Arch. mik. Anat.*, lix, 1902, p. 173).

For *staining*, Ranvier (*Traité*, p. 991) employs his gold and formic acid method.

The bichromate and silver method of Golgi may be employed with *fœtal or new-born* subjects. The *methylen blue intra-vitam* method has given good results. For the higher vertebrates the injection method should be employed. The *Encycl. mik. Technik.*, i, p. 511, recommends injection of 1 c.c. of 0·5 to 1 per cent. solution every five minutes through the vena femoralis until the death of the animal. The cochlea then to be got out, exposed to the air for fifteen or thirty minutes, and fixed for some hours (overnight) in 10 per cent. ammonium molybdate with a little osmic acid. It is then decalcified in trichloracetic acid of 5 per cent. with a trace of platinum chloride, washing for twenty-four hours and got into paraffin.

For fishes and amphibia the immersion method will suffice.

Fraser (*Ann. Otology, Rhinology and Laryngology*, xxxii, 1923,

p. 953) recommends for human material, opening the superior semi-circular canal before fixation so that the fixative shall penetrate to the internal ear. The block, which is trimmed as small as possible, is decalcified, after thorough fixation in 5 per cent. formol or Müller's fluid, by equal parts of 5 per cent. formol and 5 per cent. nitric acid, or in Perényi's solution, changing the fluid very frequently. At least one month is needed for this. Wash in water four to five days, then pass gradually through the alcohols to alcohol and ether, and imbed in celloidin (thin celloidin one month, using an evacuation pump carefully to get rid of air bubbles from the internal ear, thick celloidin one month).

1106. Other Methods. Waldeyer, Stricker's *Handb.*, p. 958 (decalcification either in 0·001 per cent. palladium chloride containing 10 per cent. of HCl, or in chromic acid of 0·25 to 1 per cent.).

Urban Pritchard (*Journ. Roy. Mic. Soc.*, xii, 1872, p. 380). Decalcification in 1 per cent. nitric acid.

Lavdowsky (*Arch. mik. Anat.*, xiii, 1877, p. 497). Fresh tissues (from the cochlea) are treated with 1 per cent. solution of silver nitrate, then washed for ten minutes in water containing a few drops of 0·5 or 1 per cent. osmic acid solution, and mounted in glycerin.

Max Flesch (*Arch. mik. Anat.*, xvi, 1879, p. 300); Tafani (*Arch. Ital. de Biol.*, vi, 1884, p. 207); Eichler (*Abh. math-phys. Cl. Sächs. Ges. Wiss.*, xviii, 1892, p. 311; *Zeit. wiss. Mik.*, ix, 1892, p. 380 (injection of blood-vessels of the labyrinth)); Siebenmann (*Die Blutgefasse im Labyrinthe des menschlichen Ohres*, Wiesbaden, Bergmann, 1894; *Zeit. wiss. Mik.*, xi, 1894, p. 386; Gray (*Journ. Anat. Phys.*, xxxvii, 1903, p. 379); Scott (*ibid.*, xliii, 1909, p. 329).

1107. Olfactory Nerve-endings, Tactile Corpuscles, etc. Besides the *gold method*, Chapter XIX, and the *methylen blue method*, Chapter XVIII, the rapid *bichromate and silver method* of Golgi should be employed, and for the olfactory mucosa gives the best results. See van Gehuchten, *La Cellule*, vi, 1890, p. 405. For *intra-epidermic nerve-endings*, besides the methods given in Chapter XL, the Golgi method should be employed. According to van Gehuchten (*La Cellule*, ix, 1893, p. 319) it gives much better results than gold methods. He uses the rapid process. For *tactile corpuscles*, etc., besides the methods given in Chapter XL, see Ramón y Cajal's neuro-fibril methods.

CHAPTER XLIV

PROTOZOA *

1108. Introduction. At the outset great emphasis must be laid on the necessity for examining all protozoa alive, and as nearly as possible in their natural conditions, whether they be parasitic or free-living forms.

For this reason it is essential, when making preparations for the microscope, to mount the protozoa in their natural medium, or one isotonic with and approaching as nearly as possible to it. Saline solutions, including Locke's and Ringer's mixtures (see appendix), are varied slightly for use with parasites and tissues of different animals.

It is also most important for the medium to have the same reaction (H-ion concentration or *p*H), *i.e.*, to be neither more acid nor more alkaline than the natural medium. White of egg added to a mounting medium for examining protozoa over long periods has the advantages of preventing bacterial growth to some extent as well as supplying a colloid.

Much advance has recently been made in methods of maintaining protozoa in artificial culture over long periods. Such cultures facilitate enormously the study of life histories, besides affording the great convenience of having a supply of the organisms at hand in the laboratory. Before dealing, therefore, with the action of any special reagents on protozoa, some of the chief methods by which they may be cultivated will be indicated.

I. CULTIVATION OF FREE-LIVING PROTOZOA

1109. Collection. Notwithstanding the wide distribution of many protozoa, it may be a matter of considerable difficulty to collect enough specimens for class or research purposes. Pelagic forms may, of course, be collected by tow-netting, etc. (See *The Microscope and its Revelation*, CARPENTER and DALLINGER, 1901, or *Fresh Water Biology*, WARD and WHIPPLE, 1918.) Sedentary and many other forms from below the surface may have to be picked out by examining weed, mud, etc., with a lens. Occasionally good temporary cultures of a required form can be obtained by keeping tow-nettings in water from the same source and supplying food; *e.g.*, we have had on several occasions, from April to June, good supplies of Stentor by leaving

* By H. P. G.

tow-nettings from the Cherwell in river water (pH approx. 8) to which wheat grains were added. The pelagic Stentor settle down, secrete houses and multiply. However, after ten days or so this dominant form is replaced by Actinosphærium, which feeds on it. The prevention of cycles of organisms, is, in fact, the chief difficulty in obtaining permanent mass cultures of any special form.

1110. Cultivation of Amœba proteus. TAYLOR (*Quart. Journ. Micr. Sci.*, 1924, pp. 69, 119) has since 1915 investigated the conditions under which this organism will complete its life history in artificial culture.

The medium used is boiled rain-water,* containing about twenty-two wheat grains to the litre (the wheat is boiled to prevent germination). The optimum reaction of a flourishing culture containing numerous large active amœbæ is about pH 6·6, and sub-cultures need only be made every three months or so. Into the new culture fluid should be carried over several cubic centimetres of the old culture, including organisms on which the amœbæ are feeding, as well as algæ, to keep the water oxygenated.

The cultures are best kept in large covered glass vessels in which the medium has a depth of 2 or 3 in., though smaller vessels may be used. They should be kept towards the back of a room away from bright light. Under these conditions the cultures can be kept with very little trouble indefinitely.

Sometimes difficulty is experienced in starting a culture owing to the presence of enemies to the amœba, such as oligochætes, ostracods, etc., and it may be necessary to make several sub-cultures to eliminate these.† If only few specimens can be found with which to start a culture, it is well to use only a small amount of medium and place it in a test-tube or Petri dish until the correct conditions are established.

Conditions which induce encystment of *A. proteus* are still being investigated. I understand from Sister Monica Taylor that these rest with the individual amœba rather than with the culture medium, and that the only way by which one can hope to encourage encystment is to starve large specimens which

* In some districts the tap-water is suitable, but here in Oxford and many other places the water is "hard." Part of this hardness (temporary hardness—so called because it can be removed by boiling) is due to the presence of carbonates of the alkali and alkaline earth metals, kept in solution as bicarbonates so long as there is CO_2 in the water. The pH may or may not be too high for the cultivation of any special protozoa to start with (here it is generally 7·6). However, when placed in shallow vessels, water containing CO_2 will tend to lose it, and consequently the water will become more alkaline (see below, § 1111, under Spirostomum).

† I understand that small rotifers, especially the red rotifer (of rain barrels), have proved themselves to be useful in small numbers as scavengers in amœba cultures.

have recently fed voraciously. She has also come to the conclusion that the lowered *p*H often found in cultures containing many cysts and young amœbæ is probably the result rather than the cause of encystment, the acidity being due to the decomposition of the residual cystoplasm of the mother amœbæ after the cysts have been liberated.

Other more elaborate methods of cultivating amœbæ have been devised by SCHÆFFER (*Carnegie Instit.*, xxiv, 1926) to imitate running water and other conditions in Nature, but for ordinary laboratory purposes the above described closed mass cultures are satisfactory.

1111. General Notes on Cultures in Aqueous Media. The acidity of a culture containing algæ can, of course, be reduced and its *p*H proportionally raised :—

1. By stirring and shaking or keeping it in a shallow vessel with a relatively large surface exposed to the air, thus enabling the CO_2 to escape.
2. By placing the culture in a bright light, since during photosynthesis CO_2 in the water is broken up.
3. By the addition of minute quantities of alkalies, such as $NaHCO_3$, NaOH, etc.

Conversely, the *p*H soon falls if the culture is made in a test-tube, where a comparatively small surface of the medium is exposed to the air, for then the CO_2 produced accumulates in the liquid; or CO_2 may be passed into the medium. (When there is only a small amount of medium in a test-tube, for example, a suitable indicator may easily be made to change colour by simply breathing into the tube (SAUNDERS, *Proc. Camb. Phil. Soc.*, i, 1925, p. 249).) Other acids may be added instead of CO_2, such as very dilute hydrochloric acid. Taylor has used tartaric acid for increasing the acidity of amœba cultures but finds that it encourages detrimental bacteria; she has had some success in eliminating undesirable organisms from cultures by temporarily making the *p*H unsuitable to them; or by adding chemicals, *e.g.*, ferrous sulphate for getting rid of a tiresome blue-green alga.

It is quite impossible to deal adequately here, with the very numerous methods devised for keeping isolation or mass cultures of various free-living protozoa, from the time of MAUPAS (*Arch. Zool. Exper.*, 1888) onwards.

Everyone knows that Paramœcium, for instance, can very easily be cultivated in hay infusions. Other media successfully used are infusions of decaying leaves, suspensions of malted milk, boiled flour water, as well as water containing wheat. The optimum *p*H is found by Saunders (*Proc. Camb. Phil. Soc.*, i, 1925, p. 249) to be 7·8–8. Taylor keeps Actinosphærium successfully in aquaria with high *p*H by feeding them on the rotifer, Chydorus or others of the numerous animals they will devour.

For *Spirostomum ambiguum*, Saunders (*ibid.*, 1924) finds media of *p*H8 and above are toxic. Therefore, unless a " soft " water is used for

a medium, the cultures must be kept in long, narrow tubes (Bishop, *Quart. Journ. Micr. Sci.*, 67, 1923, p. 405). Jenkin (*Brit. Journ. Exper. Biol.*, iv, 1927, p. 377) concludes that an increase in alkalinity above *p*H7·4 makes the body wall more permeable to water, the result being that animals swell up and burst.

Isolation cultures may be made by the " hanging drop " method and sub-cultures made daily. Woodruff kept *Paramecium aurelia* living continuously for seventeen years on the same bacterial diet. *Actinobolus radians* has been kept through 448 generations in sterile spring water with *Halteria grandinella* as food. The generations are isolated daily, the cultures being kept in small capsules. Similarly, *Spathidium spathula* was observed through 218 generations with *Colpidium colpoda* only as food (Moody, *Journ. Morph.*, xxiii, 1912, p. 349).

Woodcock uses his observation slide (hanging drop on coverslip supported on glass ring over water contained in hollow slide) (*Phil. Trans.*, 207, 1916, p. 379) for cultivating coprozoic protozoa ; they are thus provided with more air than in an ordinary hanging drop. He uses dilute broth as a culture medium.

There are numerous other media in which amœbæ, flagellates and ciliates will flourish. Such cultures are very often kept in test-tubes plugged with cotton-wool to keep them sterile, as is usual in dealing with bacteria.

1112. A Synthetic Medium. Some interesting experiments have been done by Peters (*J. Phys.*, lv, 1921, p. 1) in the direction of simplifying the medium used for cultivation of protozoa, and the following details of methods in use in the Department of Biochemistry, Oxford, have been supplied by Professor R. A. Peters (November, 1935).

The saline solution of the medium which seems most useful for growing protozoa has the following composition :—

NaCl	0·5 gm.
KCl	0·01 ,,
$CaCl_2$ anhydrous	0·02 ,,
Mg_2SO_3	0·01 ,,
AmCl	0·1 ,,
Glass-distilled OH_2	1000 c.c.

To this is added shortly before sterilising 0·3 grm. (*i.e.* 0·03 per cent.) sodium glycero-phosphate. This sodium salt, being in the form of stable crystals, is found to be more convenient than the ammonium salt originally used and is equally good so long as ammonium chloride is added to the saline solution, as above.

The ammonium glycero-phosphate used at first in the medium was in the form of a solution containing approximately 50 per cent. of this organic compound. There was no ammonium chloride, but the sodium chloride was 0·06 per cent.

Small differences in the percentages of the salts have in general little effect, and one finds, as with Ringer's and Locke's solutions (p. 731), that different workers sometimes vary the formula slightly.

The salts should be Kahlbaum (K) where possible, or other

guaranteed salts. The diluted medium should be placed in sterile test-tubes or small Erlenmayer flasks (100 c.c.), fitted with cotton-wool plugs and sterilised by steaming for one hour on three successive days as soon as possible after making up the concentrated mixture.

It is best to have pyrex tubes, but others can be used. Tubes of ordinary glass are apt to change the pH of the medium ; pH 7·3 to 7·4 appears to be the optimum for those tested.

All apparatus should be thoroughly cleaned with chromic acid, finishing with glass-distilled water. Tubes and flasks for cultures should, before filling with medium, be dried, plugged and heated to 170° C. in an air oven for an hour to sterilise.

Inoculation, using all precautions for maintaining sterility, is performed most conveniently by using a small silica pipette. Transfers to fresh tubes should be made every two to three weeks. Experience has shown that 20° C. is the best temperature for growth (Hearson's cold incubator is generally used for this purpose).

Since 1931 *Colpidium* has been grown in the Biochemical Department in a medium of approximately half the above strength containing sterile yeast.*

There is still considerable controversy as to whether ciliates such as *Colpidium*, as well as other protozoa, can live for more than two or three generations, if at all, in sterile media free from yeast or traces of some accessory growth factors.

1113. Cultures on Solid Media. Many coprozoic forms, as well as small free-living amœbæ and flagellates from soil, water, etc., will grow well on a solid agar medium prepared as for bacteriological use in test-tube slopes or Petri dishes (plate cultures). Of the many formulæ for making suitable media, that devised by Musgrave and Clegg for cultivation of amœbæ has been much used :—

Agar	2·0 grm.
NaCl	·03–·05 ,,
Liebig's beef extract	·03–·05 ,,
Distilled water	100 c.c.

Robertson (*Quart. Journ. Micr. Sci.*, 1932, p. 540) use an egg agar with Peters' medium very successfully for growing *Bodo*. She also uses a 2 per cent. agar in which is incorporated about 18 per cent. of Peters' medium for *Mastigamœba* growing with a suitable bacillus. By flooding this medium in Petri dishes with more Peters' medium "pond" cultures may be obtained in which *Chlamydophrys* or small flagellates, such as *Bodo*, flourish exceedingly.

* It would even appear that, when yeast is used, the glycero-phosphate is probably superfluous, since the Colpidium has grown well here in the Zoology Department for a time in a medium consisting of sterile yeast in Peters' saline solution only.—H. G.

Here again it has been found that when a suitable bacterium is present, *Dimastigamœba* will grow well on the agar and PETER's *saline* without the addition of a glycero-phosphate as indicated for Colpidium (see p. 567, footnote).

The material containing the amœba to be cultivated is placed on the surface of the agar in a drop of the saline, and the amœbæ as they grow will generally move away from this centre of inoculation. The bacteria or other organisms present serve as food, so long as they are not deleterious or too numerous. To guard against drying of the surface of the medium—tubes should be covered with a rubber cap or kept in a moist chamber—plates are generally inverted and some water placed in the lid of each.

After a period of division the amœbæ encyst. On transference to new media, the cysts will usually hatch and give rise to a new culture, even though they have been kept on the old medium for months or even years. The common coprozoic amœba, *Dimastigamœba* (*Nœgleria*) *gruberi*, multiplies under suitable conditions for five or six days, and then encysts. It should be sub-cultured every week or so to prevent overgrowth by other organisms.

Pure cultures of an amœba may be obtained from a single active organism or a cyst by removing one of these by the Barber technique (*Philippine J. Sci.*, B, ix, 1914, p. 307). The following simple method, devised by Drew, may be used to start a culture from a single cyst provided it be large enough to be seen with a low magnification.

1114. Separation of a Single Cyst with a Capillary Pipette. To make the pipette, take a capillary glass tube about 6 cm. long and 1 mm. in diameter. Flame the centre and draw out quickly to the fineness of a hair. Break off the two outer pieces, about 5 cm. from each end, to obtain two short pipettes, consisting of a wider portion and a very fine capillary portion.

Next select a culture of the amœbæ, rich in cysts, add a drop of sterile water, and rub a portion of the growth into this with a sterile platinum wire. Allow a minute portion of this emulsion to run into the capillary end of the prepared tube, and then run in sterile water till about 0·5 cm. of the broad portion of the tube is filled. Mix the contents of the tube by vigorous rotation.

Now prepare an agar film on a microscope slide, by melting the medium in one of the stock tubes and pouring a few drops on to a slide. Allow this to set. Place on the microscope stage and focus the upper surface with an inch objective. Tap out on to filter paper some of the liquid in the capillary tube, and then, whilst looking through the microscope, gently touch the film with the fine end of the pipette. A small volume of the suspension of cysts will run on to the jelly and will spread out in an area which is quite visible, and which occupies only a small portion of the field. If no cyst is present, or if there should be more than one,

place another drop on a fresh film and repeat till a single cyst is obtained on the film. The method is simple, and with practice one can make half a dozen such cultures in an hour. Place the slide film surface downwards above water in a Petri dish (this is conveniently done by resting it on two corks), and cover the dish. Examine day by day, till numerous amœbæ are found, and then allow them to encyst. From this culture cysts may be inoculated into an ordinary test-tube slope, and so cultures obtained of the one species of amœba feeding on any bacteria that happen to be carried over in the inoculum with it—sub-cultures being made as often as desired. Most soil amœbæ appear to feed upon almost any of the common bacteria, but there is a considerable element of luck as to whether its favourite food will be carried over at every sub-culture. It is better, therefore, to determine for which of the many organisms present the amœba, being cultivated, shows preference and then to try to obtain it in pure culture.

1115. To obtain a Culture of One Species only of Amœba feeding upon Bacteria of only a Single Species—a "so-called" Pure Mixed Culture. To a tube containing a culture in which the amœbæ have mostly encysted, add hydrochloric acid solution strong enough to kill all organisms except the encysted amœbæ. (Some soil cysts are said to tolerate 2 per cent. hydrochloric acid for twenty-four hours, but others may be killed in much less time by acid only one-tenth that strength, and preliminary experiments will be necessary. DOBELL *Parasitology*, xix, 1927, p. 288) finds that *E. histolytica* cysts can survive in $\frac{N}{10}$ HCl (*i.e.*, 0·36 per cent.) for as long as three hours at ordinary temperature, whereas $\frac{N}{20}$ kills all *active* amœbæ in ten to thirty minutes.) Then pour off the acid, neutralise with sodium bicarbonate, and thoroughly wash the cysts on the slope with sterile water. With a platinum loop scrape off the cysts and inoculate into a fresh tube of medium, adding a loopful of a dilute emulsion of the bacterium with which it is desired to cultivate the amœbæ.

Of course, precautions against infection must be observed throughout and cultures are generally kept in tubes plugged with cotton-wool. It is, however, possible to lessen the risk of contamination of plate cultures by inverting them into a little mercuric chloride solution in the lids.

II. CULTIVATION OF BLOOD PROTOZOA

1116. In all these processes it is most essential to avoid all bacterial contamination, consequently aseptic conditions must prevail throughout. For further information consult text-books on Bacteriology or Wenyon's *Protozoology*.

Malarial parasites, first cultivated *in vitro* by Bass and Johns (*Journ. Exper. Med.*, xvi, 1912, p. 567). To 10 c.c. of malarial blood from a vein add 0·1 c.c. of a 50 per cent. solution of dextrose and defibrinate. Distribute the blood in test-tubes and incubate at 40° C.

Parasites will be found in some of the red cells which settle to the bottom of the serum.

Sub-culture every few days, but normal blood treated as above may be used for the medium.

Trypanosomes, Leishmania and many other flagellates are cultivated in blood agar, of which the *N. N. N.* medium devised by Novy, MacNeal, Nicolle, is the most important. It is made by heating together :—

Water	900 c.c.
Agar	14 grm.
NaCl	6 ,,

About 5 c.c. of this mixture is put into each test-tube, which is plugged and sterilised in the ordinary way. When it has cooled to 50° C. and is still liquid, 2 or 3 c.c. of sterile rabbit's blood are added to each tube, which is rapidly revolved in order to mix the two fluids without forming bubbles. Each tube is then cooled so that the blood agar forms a sloped surface, and inoculation is made into the condensation fluid at the bottom. The flagellates multiply in this fluid but sub-cultures must be made every few days.

III. CULTIVATION OF INTESTINAL PROTOZOA

1117. The numerous media, which have recently been used for cultivating entamœbæ, ciliates and flagellates from the alimentary canal of man and other vertebrates, all consist of a saline solution containing nutrient material, such as serum, ascitic fluid or egg. They may be used liquid (*e.g.*, 1—4 below) or solid (by coagulating the egg or serum or by the addition of agar, etc.), and many of the media now most used (§§ 1119, 1120) consist of a liquid and a solid. The following are some of the most important media :—

1118. Serum-Saline-Citrate medium consists of 0·5 grm. Löffler's dehydrated blood serum in 100 c.c. distilled water, containing 0·7 grm. sodium chloride and 1 grm. sodium citrate (Andrews, *Journ. Parasit.*, xii, 1926, p. 148; Tanabe, *idem*, p. 101). To maintain *Trichomonas* from man, rat and other warm-blooded vertebrates in this medium sub-cultures have to be made every two or three days by introducing a drop of a three-day culture into a fresh tube of medium and incubating at 35° to 37° C.

Trichomonas from amphibia grow better at room temperature, and in a medium containing rather less sodium salts. To these media may be added 1 or 2 c.c. of white of egg, which exerts some bactericidal action.

Hogue's Egg Medium (*Amer. Journ. Trop. Med.*, i, 1921, p. 211). A clear medium with a granular sediment made by shaking up a whole egg in a flask with glass beads and adding 200 c.c. Locke's solution. The mixture, kept constantly moving, is heated over a water bath for fifteen minutes and then filtered through cotton-wool. Five to 6 c.c. are placed in each test-tube and sterilised in an autoclave for twenty minutes at 120° C.

In this, Hogue has obtained pure line cultures of *Trichomonas* by starting with a single individual.

Embadomonas from various sources, as well as *Trichomonas*, grow well in the medium; parasites from warm-blooded animals being incubated at 35° to 37° C., while those from cold-blooded animals grow best at ordinary room temperatures.

Amœbæ of cold-blooded vertebrates (turtle and frog) have been cultivated in a very simple medium devised by BARRET and SMITH (*Annal. Trop. Med. Parasit.*, xx, 1926, p. 85). It consists of 1 part inactivated human serum and 9 parts of 0·5 per cent. sodium chloride solution. The *p*H of this medium is 7·6 to 7·8.

Entamœba ranarum is said to grow in media of reactions between *p*H5 and *p*H10 though the optimum is between 7 and 8.

BARRET and YARBROUGH (*Amer. Journ. Trop. Med.*, i, 1921, p. 161) cultivated *Balantidium coli* in a more dilute serum, viz., 16 parts of the sodium chloride solution to 1 of serum. As a rule, however, sub-cultures had to be made every two days. See also JAMESON, § 1120, below.

Trichomonas of the human mouth and *Entamœba gingivalis* multiplied in a mixture of equal parts of ascitic fluid and Locke's solution (OHIRA and NOGUCHI, *Journ. Exper. Med.*, xxv, 1917, p. 341). See also DOBELL, § 1120 below.

Herpetomonas from flies' intestines are best cultivated (DRBOHLAV, *Journ. Parasit.*, xii, 1926, p. 188) in dilute blood agar provided the *p*H is low—5·6 to 6·4.

1119. Bœck's Locke-Egg-Serum Medium (L.E.S.) and various modifications devised by BŒCK and DRBOHLAV (*Amer. Journ. Hygiene*, v, 1925, p. 371) for the cultivation of intestinal protozoa all consist of two parts: a solid and a liquid medium; the former is prepared in the form of an ordinary bacteriological slope and is more or less covered by the liquid.

In the original formula the *solid medium* consists of an emulsion of eggs in Locke's solution (four whole eggs to 50 c.c. solution) coagulated by heating to 70° C. and sterilised in an autoclave at a pressure of 15 lb. for twenty minutes. The liquid medium is a mixture of 1 part inactivated human serum to 8 parts of sterile

Locke's solution—sterilised by filtration, if necessary. The medium thus prepared and warmed to the required temperature is inoculated with the material containing the organisms to be cultivated. This is introduced at the junction of the solid and liquid medium at the bottom of the tube by means of a sterile capillary pipette, and it is in this region that the organisms chiefly develop when incubated at 37° C.

The medium has an initial reaction of *p*H7·2—7·8, which is suitable for the growth of amœbæ. However, owing to the growth of acid-forming bacteria in the presence of the glucose of the Locke's solution,* the medium soon becomes too acid for continued growth and sub-cultures have to be made every two or three days.

Many modifications of this medium have been devised by BŒCK and DRBOHLAV, one being the use of ordinary blood agar (or *N.N.N.*) for the solid medium, and for the liquid, dilute egg white (the white of one egg to a litre of Ringer's solution).

1120. For the **cultivation of Entamœba histolytica** and some other entozoic protozoa, valuable improvements have been made in the constitution of this L.E.S. medium by DOBELL and LAIDLAW (*Parasit.*, xviii, 1926, p. 283). Also DOBELL (*Parasit.*, xx, xxiii, xxvi). They recommend making up the medium with Ringer's solution † and the introduction of solid rice starch to replace the carbohydrate (glucose in the Locke's solution) of the L.E.S. The very small grains of this form of starch are rapidly ingested by the amœbæ, which multiply enormously and often finally store up glycogen in their protoplasm, and encyst.

Blastocystis, which causes much trouble in most cultures of entozoic protozoa, can be eradicated by the presence of the starch, provided that there are no starch-splitting bacteria present, and these, the authors find, can usually be eliminated by adding flavine (1 in 20,000) to the culture. The starch should be dry and sterilised by heating it, loosely packed in small tubes, to a temperature of 180° C. It is introduced into the medium by a platinum loop or spatula, and falls in a little heap to the bottom of the tube, and the amœbæ are inoculated on to its surface.

After trying many different forms of the double medium, they find that the richest and most prolonged growths of amœbæ are obtained on the one having coagulated horse serum as the solid constituent and Ringer egg-white as the liquid, together with rice starch.

To prepare this medium, " HS re + S," suitable volumes of whole horse serum (sterilised by filtration) are placed in tubes, with all aseptic precautions, and set in the form of ordinary slants by heating in an inspissator at 80° C. It is most important not to overheat, for the

* Owing to the numerous slight modifications now given in the formulæ for these fluids, it is necessary to state that the Locke's solution used by Bœck and Drbohlav contained 9 grm. NaCl, 0·4 grm. KCl, 0·2 grm. $CaCl_2$ and $NaHCO_3$, 2·5 grm. glucose in a litre of distilled water.

† The Ringer solution used by Dobell and Laidlaw contained 9 grm. NaCl, 0·2 grm. KCl, and 0·2 grm. $CaCl_2$ in a litre of distilled water. For other fluids, see § 1430.

serum itself appears to act as an effective buffer. The egg-white is stronger than that used by Drbohlav, being the white of four eggs to a litre of Ringer's solution ; one great advantage of this stronger solution is the ease with which films of the amœbæ may be fixed for cytological study.

In this medium *E. histolytica* and *E. coli* usually live seven to ten days, but *E. gingivalis* has to be transplanted into fresh tubes every five or six days.

Endolimax nana grows better in the original L.E.S. medium, since this parasite does not need starch.

Trichomonas grows very readily in the medium "HS re + S" DOBELL (*Parasit.*, xxvi, 1934, p. 531) and *Balantidium Coli* according to JAMESON (*Parasit.*, xix, 1927, p. 419).

Many intestinal protozoa will grow in simpler media, and DOBELL (p. 538) suggests that the following might be a good double medium for use in laboratories where serum is not easily obtainable, "Slopes of heat-coagulated egg-white, covered with unheated egg-white diluted with RINGER's fluid."

EXAMINATION OF LIVING PROTOZOA. QUIETING OF VERY ACTIVE CILIATES AND FLAGELLATES

1121. Mechanical Methods. (*a*) Entangling them in the meshes of cotton-wool, mycelial threads of a fungus, etc.

(*b*) Increasing the viscosity of the mounting medium by adding (i.) *gum*. EISMOND (*Zool. Anz.*, xiii, 1890, p. 723) added a drop of thick aqueous solution of cherry-tree gum to the water containing the organisms. LYON (*Amer. Journ. Physiol.*, xiv, 1905, p. 427) found gum arabic solution neutralised with caustic soda perfectly successful. CERTES (*Journ. Roy. Micr. Soc.*, 1891, p. 828) added an intra-vitam stain (methyl blue or violet dahlia, No. 170) to the gum solution. (ii.) Gelatine. JENSON (*ibid.*, 1892, p. 891) added 1 drop of a 3 per cent. solution, warmed, to one of water containing the organisms giving a concentration of 1·5 per cent. (iii.) A dilute solution (about 1 per cent.) of agar may be used in the same way as gelatin, and an intra-vitam stain introduced as well, if desired.

1122. Narcotisation Methods. *Cocaine* solutions being unstable, the best results are obtained by placing a minute crystal in the mounting medium under the coverslip and allowing it to diffuse gradually. Myonemes can be clearly made out in this way, and the working of the excretory system in Paramœcium, for example. A freshly prepared 0·1 per cent. solution added in the proportion of 1 to 10 of medium (*i.e.* making a 0·01 per cent. solution) required two or three hours to prevent the stalks of Vorticellids from contracting. Nearly all the peristomes had by then closed, so that this method was not much good for preparing these forms for fixation in the expanded form ; the same applies to other narcotics tested.

Eucain hydrochloride is stable in aqueous solution and a 1 per

cent. solution is said (HARRIS, *ibid.*, 1900, p. 404) to be better for Vorticellids, but we have had no success in narcotising *Zoothamnium* with their oral cilia extended.

Menthol is best used by placing a minute crystal under the coverslip with the protozoa, but maceration follows very speedily on cessation of movement in the few ciliates tested.

Chloral hydrate may be used in 1 per cent. solution or in solid form, but appears to be little better than menthol for ciliates.

Chloroform water, coloured with neutral red, has proved in our hands as useful as any narcotic for quieting active ciliates and flagellates on the slide. One drop of chloroform rubbed up with 6 to 10 c.c. of a dilute neutral red solution will make a homogeneous mixture. The protozoa are best mounted in as little fluid as possible, and a minute drop of the mixture placed to one side of the coverslip with a thin glass rod, and allowed very gradually to diffuse in. When this is done successfully the protozoa will move away, and after some minutes, the ones situated about the middle of the preparation will be moving slowly only and the working of the cilia, contractile vacuoles, etc., will be clear, while the protozoa are very little, if at all, distorted. Amytal is used by LUND (*Journ. Morph.*, lviii, p. 260). Refer also to § 13 where further information is given.

1123. Addition of Dyes. *Intra-vitam Stains.* Strictly speaking, all stains are to some extent toxic and consequently slight excess of an intra-vitam stain will more or less check the movements of active forms. To obtain good results the solutions used for intra-vitam work must be extremely dilute and, moreover, dilution must, of course, be effected so far as possible with the liquid in which the organisms are living. The strengths used generally vary between 0·01 and 0·0001 per cent. The dye employed must be as pure as possible. Those generally used for protozoa are : neutral red, methylen blue, Bismarck brown, toluidin blue.

For *mitochondria* of protozoa FAURÉ-FREMIET (*Arch. d'Anat. micr.*, xi, 1910, p. 457) and others use janus green, violet dahlia, crystal violet. BRANDT (*Biol. Centrbl.*, i, 1881, p. 202) recommended " a dilute solution of hæmatoxylin " as an intra-vitam stain. TAYLOR (*Univ. Calif. Publ. Zool.*, xix, 1920, p. 417) used a 0·0001 per cent. solution for organelles and fibres of ciliates. Even a 0·5 per cent. solution in water added to cultures rich in flagellates and ciliates is very little toxic to the protozoa, but the same may be said for many other dyes, *e.g.* indigo-carmine.

Trypan blue, another intra-vitam stain, is taken up by many tissues. A solution (1 to 2 per cent. in sea water) is recommended by Dr. Lebour (personal communication) for staining the plates of thecate dinoflagellates. The theca is stained a clear pale blue and the divisions between the plates show up as dark lines. The dinoflagellates are, however, killed and, owing to their extreme

delicacy, it is unlikely that they could be kept alive even were the solution used more dilute.

Ink (Stephens' blue-black) is excellent for staining the epispores of gregarine and other spores to show up their processes (*Quart. Journ. Micr. Sci.*, 61, 1915, p. 83). A half-saturated solution of *methyl blue* in distilled water is also very good for the same purpose and less likely to cause a precipitate when host tissue or fluids are present.

CERTES (*Bull. Soc. Zool. de France*, xiii, 1888, p. 230) recommends examination of organisms in coloured media, in which they do not stain but show up on a coloured background, saying that infusoria will live in a solution of anilin black for weeks. FABRE-DOMERGUE (*Ann. de Microgr.*, ii, 1889, p. 545) uses in this way a concentrated solution of diphenylamin blue.

For preparing coverslips for neutral red staining see under Blood, § 872. For a general discussion of the whole subject refer to Chapter XXXI, §§ 739 to 775.

1124. Demonstration of Cilia and Flagella. Any of the above methods which slow down the rate of movement of the ciliates and flagellates will tend to show up the cilia or flagella by which they move (see also the effect of iodine below).

Tannin (tannic acid), the solution recommended by WADDINGTON (*Journ. Roy. Micr. Soc.*, 1883, p. 185) for demonstrating cilia of Paramœcium, has, in my hands, exactly the same effect as, judging by his figures, it had in his, viz. the extrusion of trichocysts. With very dilute solutions some trichocysts are shot out, and often they are so thick that the cilia are hidden altogether.

Sulphurous acid. An alcoholic solution was recommended by Waddington (see above) for showing up cilia. It is useful also for reducing the activity of ciliates. I cannot find, however, that an alcoholic solution is any better than an aqueous one, e.g., equal volumes of normal sodium bisulphite and hydrochloric acid solutions coloured with neutral red.

1125. Toxic Mounting Media. Useful observations may often be made on protozoa by adding to them gradually (by running in under the coverslip) or mounting them direct in certain toxic media.

Iodine is especially serviceable in this way. It is used in watery solution (*i.e.* dissolved in a solution of potassium iodide) of a light sherry colour. Lugol's solution is a suitable strength. *Cilia and flagella* generally show up very clearly, being temporarily fixed by the iodine. Any *starch* present is, of course, stained blue, and *glycogen* brownish; the latter being somewhat soluble in water, will generally show a blurred edge.

For the examination of fæces for protozoa and their cysts, it is a great help to have preparations mounted in iodine solution as well as in normal saline. Routine examination for diagnostic

purposes should be carried out as follows (Wenyon, *Lancet*, ii, 1915, p. 1173): From a suitable part of the specimen sent to the laboratory take a minute portion on a platinum loop and rub it into first a small drop of normal saline and then one of iodine solution on slides and cover each with a coverslip. It is necessary to examine several preparations from different regions of a specimen before it can be diagnosed as free from any special protozoa.

Osmic Acid. One per cent. solution may be used to kill and fix protozoa while actually under observation, and may be followed by such a stain as picro-carmine.

Methyl green acetic acid mixture may also be allowed to diffuse into a preparation while under observation.

A saturated solution of methyl green in 0·5 to 1 per cent. of acetic acid is an important stain for showing up the nuclei of protozoa. It is a chromatin stain for fresh (unfixed) cells. Refer also to §§ 621, 623.

FIXING AND PRESERVING FOR PERMANENT PREPARATIONS

1126. Films and Smears. *When films are mentioned in the following notes, it is to be understood that a thin layer or smear of the material on a coverslip is meant.* The making of these requires much practice and care. The protozoa or other material must be sufficiently spread out to give complete transparency, and the films must never be allowed to dry, for then the protozoa would be distorted; on the other hand, if too wet, most of the material will wash off in the fixative.* When just sufficiently dry, the coverslip is dropped, film downwards, on to the surface of the fixative already prepared in a watch-glass. Minchin (*Quart. Journ. Micr. Sci.*, liii, 1909, p. 755) elaborated a process of dealing with these films by passing them through the different reagents in solid watch-glasses or capsules. The upper side of the cover-glass is kept clean throughout so that at any stage the process can be controlled by examination under the microscope. Curved forceps are useful for transferring the coverslips from one watch-glass to another; since they float on water, the films can be easily washed in running water, if desired. The other reagents are best introduced by means of a pipette underneath the coverslip while its corners rest on the watch-glass.

For most protozoa, this is the best way to deal with them whole, but for blood parasites, a rougher method may be used as well, by spreading out the blood on slides. These preparations.

* In making films of free-living flagellates and ciliates it is generally necessary to add a trace of gelatin water or Mayer's albumen (§ 209) to help them to adhere to the cover-glass. With parasites there is usually enough albumen from the host fluids to answer the purpose.

similar to those used in Bacteriology, are well referred to as *smears* to distinguish them from *films* on coverslips. They may be allowed to dry during fixation (see Osmic Vapour, § 1144, below), and are well stained with Giemsa, § 1155. The drying causes the protozoa to flatten out somewhat and therefore to appear rather larger, which may be a great advantage in dealing with small flagellates and their flagella, so long as distortion is not too great. Wet fixation, on the other hand, always tends to produce shrinkage but gives a more reliable representation of the organism.

1127. Free Protozoa. The older method of treating protozoa while mounted between coverslip and slide is still sometimes used, especially for free-living forms—the various reagents being run in under the coverslip by means of pipettes and filter paper (Minchin, *Nat. Sc.*, iii, 1893, p. 112; Doflein, 1916, p. 376).

When the protozoa are abundant they may, of course, be treated *en masse* by pouring into comparatively large volumes of fixative in a capsule or centrifuge tube and allowing them to settle. The subsequent processes of washing, staining, and dehydrating may be hastened by centrifuging slowly. To clear, pour clove oil down the side of the tube containing the protozoa in absolute alcohol. They will soon sink to the bottom, and can be taken up with a wide pipette and distributed on slides as required, and mounted (Doflein, p. 380).

1128. Sections. When protozoan parasites in tissues are to be dealt with, it is necessary to determine the true relationship of parasite to host by fixing with as little disturbance as possible small pieces of the tissue taken from the host immediately after death. The tissue is then imbedded for sections.

1129. Imbedding Protozoa (Minchin, *Quart. Journ. Micr. Sci.*, lx, 1915, p. 508). A thin slice of a block of amyloid liver, preserved in alcohol, is placed in a shallow glass vessel with a flat bottom, containing alcohol to the height of about 1 cm. The dish is placed on the stage of a dissecting microscope. The objects to be imbedded are taken up in a pipette from 90 per cent. alcohol, after fixation, and placed a few at a time on the slice of liver and orientated as desired. A tiny drop of glycerin albumen solution is taken up on the point of a needle and caused to touch the surface of the alcohol immediately above the small objects. The dense albumen solution falls at once through the alcohol and spreads out over the objects on the liver; at the same time the glycerin is extracted and the albumen coagulated by the alcohol, with the result that the objects are stuck on to the liver. When a sufficient number has been attached in this way the piece of liver is trimmed, if necessary, and imbedded in the usual way. See also § 157 for other methods.

For the ordinary procedure of dehydrating, clearing, imbedding, etc., reference may be made to p. 723 for students.

COMMON FIXATIVES FOR PROTOZOA

1130. A saturated solution of mercuric chloride ($HgCl_2$ or corrosive sublimate) in distilled water is contained in numerous fixatives, and will be referred to below as sublimate solution. Unless otherwise stated, films should be fixed for ten to thirty minutes, and pieces of tissue one-half to several hours, according to size. Most fixatives may be well used warm (40° to 42° C.) to increase penetration, and sometimes even hot. All preparations so fixed require thorough washing in 70 per cent. alcohol to remove the excess of mercury. This may be facilitated by adding a little iodine to the alcohol used for washing until it is no longer decolourised. Care must be taken to remove all iodine as well as mercury before attempting to stain. This may speedily be done with sodium thiosulphate solution, but as a rule 70 per cent. alcohol, changed once or twice, is all that is necessary. Tissue is often washed after being cut into sections. Films and tissues are all the better for being left in 90 per cent. alcohol for some hours to harden, the preparations being then less likely to undergo maceration or shrinkage during staining.

1131. Sublimate-Acetic. One to five per cent. of glacial acetic acid added to the sublimate solution.

1132. Schaudinn's Fluid (*Zool. Jahrb. Abth. Anat.*, xiii, 1900, p. 211). Two parts sublimate solution to 1 part absolute alcohol, with, if desired, a trace of glacial acetic acid.

Woodcock (*Phil. Trans.*, ccvii, 1916, p. 379) and Wilson (*Univ. Calif. Pub. Zool.*, xvi, 1916, p. 244) recommend the addition of 5 per cent. glacial acetic acid. Langeron (1913) shows that the alcohol used need not be stronger than 90 per cent.

This is a very important fixative for protozoa. It may be used cold or warmed to 60° or 70° C., when it is more penetrating and, therefore, requires rather less time.

Maier's modification: distilled water, 200 c.c.; absolute alcohol, 100 c.c.; sodium chloride, 1·2 grm.; $HgCl_2$, 10 grm. Ten minutes is long enough to fix thin films, it was found by Minchin (*Quart. Journ. Micr. Sci.*, lx, 1914, p. 502) to be excellent for fixing trypanosomes in films or in the mid-guts of fleas, though not so good as Flemming's fixative for the tissues. He attributes this to unequal penetration, surmising that the alcohol diffuses into the tissues and fixes them defectively before the sublimate can reach them.

1133. Sublimate-Formol. Bouin's formula (*Arch. Biol.*, xvii, p. 211, 1900), § 117.

Carleton's formula (*Hist. Technique*, 1926, p. 37)—1 part of formol to 9 of sublimate solution.

Penetrating and useful for cysts and tissues as well as for free protozoa.

1134. Sublimate-nitric. Petrunkewitsch and Gilson's mixtures (§ 74) may be used warm to fix spores and cysts.

1135. Sublimate-picric mixture recommended by YOCUM (*Calif. Publ. Zool.*, xviii, 1918, p. 342) :—

Mercuric chloride	2 grm.
Picric acid	1 ,,
95 per cent. alcohol	110 c.c.
Ether	20 ,,
Acetic acid (glacial)	20 ,,
Formol (40 per cent. H·CHO) . .	50 ,,

Such a mixture of oxidising and reducing agents would not be likely to keep. Presumably the formol is only added just before use.

TAYLOR uses this fixative hot for *Euplotes* (*ibid.*, xix, 1920, p. 417), and then stains with Mallory's triple stain hæmatoxylin or for demonstrating the fibrillar apparatus. HAMMOND (*Quart. Journ. Micr. Sci.*, lxxix, 1937, in press) also uses it for this purpose, but prefers simpler sublimate mixtures before silver impregnation methods for showing up silver line systems in ciliates.

1136. Sublimate-bichromate. ZENKER's mixture (§ 78) is said by DOBELL and O'CONNOR (*Intestinal Protozoa of Man*, 1921, p. 138) to give excellent results with intestinal protozoa, free or in tissues, but often fails to penetrate cysts properly.

If a piece of tissue is large enough to require several hours' fixation, it will have to be washed for several hours in gently running water to remove excess of bichromate before removing the excess sublimate in the usual way.

1137. Bichromate-acetic. TELLYESNICZKY's formula (§ 57) is used by DEHORNE (*Arch. Zool. Expér.*, lx, 1920, p. 47) to fix Paramœcium and Colpidium.

1138. Picro-formol-acetic or **Bouin's Fluid,** § 115.

Fix films ten to forty minutes; tissues for not more than twenty-four hours. Wash out with 70 per cent. alcohol.

A very good fixative for protozoa and tissues, but it sometimes fails to penetrate cysts and spores.

DOBELL suggested substituting a saturated solution of picric acid in 90 per cent. alcohol for the aqueous solution in Bouin's formula, and adding 1 to 2 drops of chloroform just before use to facilitate penetration of chitin. HOARE (*Parasit.*, xv, 1923, p. 374) found this modification of the fixative excellent for sheep keds containing trypanosomes. He kept the insects in the fixative for twenty-four hours (one hour warmed on the incubator, twenty-three at room temperature). He then placed them in 90 per cent. alcohol for seven days (changing the alcohol from time to time).

1139. DUBOSCQ-BRASIL modification, or alcoholic Bouin:

Alcohol (80 per cent.)	150 c.c.
Formol	60 ,,
Glacial acetic acid	15 ,,
Picric acid	1 grm.

is more penetrating, and therefore better for fixing arthropods containing parasites; also cysts, etc., especially when used warm.

GRASSÉ (*Arch. Zool. Expér.*, lxv, 1926, p. 349) recommended McClung's modification of Bouin's mixture for flagellates (§ 115).

For other protozoa urea is said to have little or no value.

1140. Hollande's Cupro-picro-formol-acetic Mixture is also said to be good for flagellates, and to be more penetrating than ordinary BOUIN (*Arch. d'Anat. micr.*, xviii, 1921, p. 96):

Picric acid	4	grm.
Neutral copper acetate	2·5	,,
Formol	10	c.c.
Glacial acetic acid	1·5	,,
Distilled water	100	,,

1141. Carnoy's Mixture (§ 90). Fix films ten to fifteen minutes, blocks of tissue a quarter to one hour, and wash out in 90 per cent. alcohol.

This mixture, one of the most penetrating fixatives known, is excellent for showing up the chromosomes of even large protozoa mounted whole, since it dissolves many cytoplasmic inclusions without destroying chromatin. It is a good fixative for glycogen, which is precipitated and can then be well shown up with Best's stain, § 670.

1142. Flemming's Fluid (§ 44). Fix thin films for about ten minutes. Penetration is very poor; therefore pieces of tissue must be small—fix one to twenty-four hours and wash in running water for about half as long before dehydrating.

MINCHIN (*Quart. Journ. Micr. Sci.*, lx, 1914, p. 506) found this the best fixative for stomachs of fleas full of rat blood and trypanosomes; the histology of the wall was extremely good and the blood not shrunk away from it, and both free and intracellular trypanosomes well preserved.

1143. Flemming without Acetic. For various modifications of Flemming for fixing the cytoplasm and its inclusions without destroying mitochondria, etc., see § 693. For other similar fixatives, see under Cytology, § 679.

1144. Osmic Vapour. An excellent fixative, to which films on coverslips may be exposed. It is used chiefly by protozoologists for smears of minute flagellates and other parasites, especially in blood.

MINCHIN used the following method for smears (*ibid.*, liii, 1914, p. 755):—

In a suitable glass slide tube place some pieces of glass rod, on which the slide can rest when the tube is tightly closed with a stopper. Into the bottom of the tube put 20 drops of 4 per cent. osmic acid solution with 1 drop of glacial acetic acid. This tube, if kept in the dark and tightly closed, can be used repeatedly.

To fix the smear, place the slide in the tube and close for ten

seconds. Then remove slide, which should be now dry, and place it in absolute alcohol for ten minutes. It may then be allowed to dry or be placed at once in Giemsa's stain (see below).

Osmic acid solution may also be used as a fixative (see § 40).

1145. Iodine is well known to be an excellent fixative (§ 88). E. S. Goodrich (*Quart. Journ. Micr. Sci.*, lxiv, 1919, p. 38) modifies Kent's method by following the dilute iodine in potassium iodide solution with a definitive fixative, such as Bouin's, and obtains excellent results with leucocytes of invertebrates. Good results have also been obtained with free-living amœbæ, ciliates and flagellates by this method, especially when fixing them *en masse* by the centrifuge method, § 1127.

The two fixatives may be mixed before use, *e.g.* 1 drop of Lugol's iodine solution to about 10 of Bouin's fluid makes a very satisfactory mixture in which to fix films.

CHIEF STAINS USED FOR PROTOZOA

1146. A. *Hæmatoxylin.* For most protozoa iron hæmatoxylin staining is unsurpassed.

(*a*) Heidenhain's **Iron Hæmatoxylin.** Use, as a mordant, a dilute solution of iron alum in distilled water (about 3·5 per cent.). As stain, 0·5 per cent. ripened solution of hæmatoxylin in distilled water.

To ripen the solution, allow to stand for some weeks until the reddish solution becomes deep brown owing to some hæmatoxylin being oxidised to hæmatein. Dobell and O'Connor recommend putting it " in a flask plugged with cotton-wool, in a warm place—if possible, in sunlight—and shaking from time to time." See also p. 162.

Mordant films or sections taken from distilled water for six hours or more—overnight is a suitable time. Rinse in distilled water and place in the stain for a similar time. Then again rinse the preparation—now black—in distilled water, and differentiate by washing out the stain in the iron-alum solution, diluted if necessary. The result should be checked and controlled by examining under the microscope from time to time.

The preparation should be greyish, due to the chromatin being black. Wash it in distilled water and then in running tap-water, for at least half an hour.

The mordanting and staining may be shortened to two or three hours each, but then the chromatin will only stain blue, instead of black as by the longer method, or alcoholic solutions may be used and the time shortened still more.

(*b*) Mallory's **Ferric Chloride Hæmatoxylin** (*Journ. Exper. Med.*, v, 1900, p. 18). Use as mordant a 10 per cent. aqueous solution of ferric chloride, and as stain a freshly prepared 1 per cent. solution of hæmatoxylin. Mordant films or sections for

three to five minutes, drain and stain in excess of hæmatoxylin for the same time. Wash and differentiate in very weak solution of ferric chloride (0·25 per cent.). Wash well in tap-water to stop decolourisation at the correct moment as determined by examining under the microscope.

Chromatin dark blue, fibrin greyish, red blood corpuscles (after fixing in Zenker), greenish-grey, connective tissue tinted pale yellow.

(*c*) WEIGERT's **iron-hæmatoxylin** mixture, in which the mordant is already mixed with the stain (§ 284) affords a rapid method, requiring only a few minutes and no differentiation. DOBELL and O'CONNOR find it specially good when the protozoa are more or less imbedded in mucus.

(*d*) DOBELL's **alcoholic iron-hæmatein** method (*Arch. Protistenk.*, xxxiv, 1914, p. 139) requires only a short time. Transfer films or sections from 70 per cent. alcohol into 1 per cent. iron alum in 70 per cent. alcohol and leave them to mordant for ten minutes, rinse in 70 per cent. alcohol and place in 1 per cent. hæmatein in 70 per cent. alcohol for ten minutes. Differentiate in iron-alum solution or acid alcohol (70 per cent. containing 0·6 per cent. HCl). Then wash in several changes of 70 per cent. alcohol.

This stain, though good for chromosomes, is said by Dobell to be unreliable for staining cysts owing to unequal penetration.

(*e*) DELAFIELD's **hæmatoxylin** (see § 295) is a good stain for protozoa mounted whole.

Transfer films and sections from distilled water into a very dilute, slightly acidified, solution of the stain. For progressive staining five or ten minutes is generally enough (or the preparation may be treated regressively, *i.e.* overstained by leaving longer and differentiated in 0·5 per cent. HCl in water). The preparations will be pink. Wash until blue, *i.e.* alkaline, in tap-water. Assuming that the tap-water is alkaline; if not, a trace of sodium bicarbonate may be added to it.

N.B.—Care should be taken that the clearing and mounting media are quite neutral. Any acidity will, of course, turn the preparation red again and in time decolourise it.

(*f*) EHRLICH's **hæmatoxylin** (acid) (see § 296) is also useful for protozoa and tissues. Use pure, undiluted, otherwise proceed as for DELAFIELD's stain (see above).

(*g*) MAYER's **hæmalum** (see §§ 288 and 289), recommended by DOBELL (*Amœbæ in Man*, 1919, p. 6) as a reliable and rapid stain for cysts of protozoa, should be a deep red colour; when it turns brown and precipitates, it is no longer fit for use. Transfer films or sections from distilled water to the stain for five to twenty minutes, then wash and mount as described above for DELAFIELD's hæmatoxylin.

(*h*) MAYER's **glychæmalum** (see § 291) is similar to Ehrlich's

hæmatoxylin and may be used in the same way. CHATTON uses it for peridinians after picric acid fixative (*Arch. Zool. Expér.*, lix, 1920, p. 21), and KEILIN (*Parasit.*, xv, 1923, p. 103) for gregarines.

(B) *Carmine* stains are penetrating and useful for staining protozoa, especially in blocks of tissue, before imbedding.

1147. (*a*) GRENACHER'S **borax carmine** (see § 271) contains about 35 per cent. alcohol and is alkaline, and may, therefore, harm delicate objects. DOBELL (p. 6) says that "used warm, and acidified with a small quantity of glacial acetic or hydrochloric acid, it will often stain the contents of cysts when all other methods have failed." One would expect, since the acid and borax are incompatible, that paracarmine (see below) diluted with an equal volume of water would be equally satisfactory.

(*b*) MAYER'S **paracarmine,** made up in 70 per cent. alcohol (§ 273), may be diluted and slightly acidified, for staining whole, especially large, protozoa. It is generally better to stain overnight and wash out with acid alcohol, but sometimes twenty to thirty minutes will be enough to give a good colour by the progressive method.

(*c*) **Alum carmine,** aqueous solution (see § 254), is useful for staining whole protozoa, also picro-carmine, § 266.

(*d*) HOLLANDE'S **iron-chloro-carmine** (see § 259). A very intense stain suitable for mitochondria and other cytoplasmic inclusions in sections of protozoa after suitable fixation.

1148. HICKSON'S **iron Brazilin stain** (*Quart. Journ. Micr. Sci.*, xliv, 1901, p. 469) may be used for protozoa after fixing in Schaudinn's fluid for fifteen minutes and washing in 70 per cent. alcohol for at least an hour.

Mordant for one to four hours in 1 per cent. iron alum in 70 per cent. alcohol, rinse in 70 per cent. alcohol and stain three to sixteen hours in 1 per cent. brazilin in 70 per cent. alcohol, then wash thoroughly in several changes of 70 per cent.

According to CARLETON (p. 299) brazilin requires partly oxidising into brazilein to give precise staining.

1149. **Safranin O,** especially after Flemming's fixative, is useful for parasites, free or in tissues (see § 372).

A solution in absolute alcohol is generally diluted with about the same volume of water, or a saturated solution in water may be used for a few minutes. The stain becomes brick-red when subsequently treated with picric acid (see § 1158, below). It may be differentiated with acid alcohol, picric acid, or clove oil.

1150. **Acid fuchsin** (Magenta S, etc.; see § 319) is generally used in aqueous solution (0·5 per cent. to saturated) for a few minutes. Preparations can be differentiated in tap-water, since the stain easily washes out in alkalies.

1151. **Counterstains.** Many stains, such as **eosin, orange G, light green, Bordeaux red,** dissolved in 96 per cent. alcohol, are

used as simple counterstains after nuclear stains such as the above. Sections and films are placed in them for a minute or two before final dehydration in absolute alcohol. They may be used dissolved in weaker alcohols, if desired, and eosin, frequently used after hæmatoxylin, may also be used in 1 per cent. aqueous solution. Eosin may be removed from tissues to some extent by prolonged washing in tap-water owing to its solubility in alkalies.

For staining protozoa in tissues, double or triple stains are specially useful.

Light green and picric acid is recommended by Minchin as a double counterstain. (1 grm. of "licht grün" and 0·5 grm. picric acid are dissolved in 100 c.c. 90 per cent. alcohol.) This stain or light green alone gives especially good results after safranin, magenta or carmine.

Eosin and light green is recommended by Chatton (*Arch. Zool. Expér.*, lix, 1920, p. 21). This is a simplification of Prenant's method (*Arch. d'Anat. micr.*, vii, 1905, p. 430), as follows : Alcohol (95 per cent.) is saturated with eosin W.G. and light green F.S. This solution keeps indefinitely.

Sections are stained for five minutes. They are then rose, and are differentiated in absolute alcohol containing 5 per cent. of acetic acid until the connective tissue of vertebrates or the chitin of arthropods is green. The preparations are then washed in xylol and mounted in neutral balsam. The method is said to be particularly useful for the study of parasites in Copepods.

Picro-nigrosin, a mixture of 1 part of saturated aqueous solution of nigrosin with 9 parts of saturated aqueous solution of picric acid, is used as follows : Transfer films or sections from distilled water into the stain for five to seven minutes, wash in tap-water, then rinse rapidly in 70 and 90 per cent. alcohol. Complete dehydration and mount. Connective tissue and chitin should be blue and muscles yellow. Very pretty preparations may be obtained after carmine. The method works especially well after sublimate fixation, it is not so successful after Bouin's fluid.

Alcoholic solutions of this stain are sometimes recommended.

Indigo-carmine solutions may be made up in many ways. A saturated solution in 70 or 90 per cent. alcohol is satisfactory but a much stronger solution may be made in water.

Picro-indigo-carmine mixtures may also be made in a variety of ways. Three parts of a saturated solution of indigo-carmine to 1 part of a saturated solution of picric acid, both in 70 per cent. alcohol, makes a good counterstain.

Ramón y Cajal's uses a watery solution (§ 426) after carmine or magenta.

Borrel (*Annal. Inst. Past.*, xv, 1901, p. 57) recommends a mixture of 2 volumes of a saturated watery solution of indigo-carmine with 1

volume of a saturated solution of picric acid, and staining for five minutes, after magenta 1 per cent. (aqueous), one hour.

Borrel fixes in OsO_4, 2 grm., platinum chloride 2 grm., chromic acid 3 grm., glacial acetic 20 c.c., water 350 c.c., for twenty-four hours. Then running water for some hours. Stained slides are differentiated in absolute alcohol and then oil of cloves.

Dobell (*Amœbæ in Man*, 1919, p. 7) says he obtained excellent results by using Borrel's method after safranin with sections containing *E. histolytica.*

Hoare (*Parasit.*, xv, 1923, p. 357), uses, for sections of ked's gut containing trypanosomes, Heidenhain's iron hæmatoxylin and counterstains by Ramón y Cajal's magenta and picro-indigo-carmine).

DOUBLE AND TRIPLE STAINING METHODS

1152. Mann's **methyl-blue-eosin** mixture (§ 356). This mixture keeps indefinitely and can be used repeatedly.

Transfer films or sections from distilled water (on no account from alkaline liquids, such as tap-water, since methyl blue is insoluble in alkalies as well as in alcohol, and eosin is soluble in both) to the mixture for five to ten minutes, then wash in water. In distilled water both dyes wash out slightly, but in tap-water only the eosin. Dehydrate rapidly and mount.

If used for fresh material, it is best to dilute the solution about ten times. It may be used after most fixatives, especially those containing sublimate or chromic acid; also as a counterstain after much-washed-out iron hæmatoxylin.

Mann's long method comprises staining for twelve to twenty-four hours, rinsing in distilled water for half a minute, thoroughly dehydrating in caustic alcohol (see Mann's "Histology," 1902, p. 217).

Chatton's modification of Mann's stain (*Arch. Zool. Expér.*, lix, 1920, p. 22), recommended for sections after fixation with picric acid mixture is to use distilled water saturated with methyl blue and eosin W.G., a mixture of equal volumes of the two saturated solutions appears to be satisfactory. The mixture keeps indefinitely.

Stain sections for fifteen minutes—they will be violet—pass them quickly into water, 95 per cent. alcohol, and absolute alcohol, the latter containing 1 drop of ammonia per 10 c.c. They should now be rose. Differentiate in clove oil while examining under the microscope and, if necessary, return to the akaline alcohol.

Chitin, connective tissue, nuclear chromatin, not associated with plastin should be blue, while muscles, nucleoli, caryosomes remain brilliant rose.

Dobell's modification of Mann's method is to stain for four to twelve hours and, after washing in distilled water, to differentiate in 70 per cent. alcohol containing a little orange G. (A few drops of a saturated solution added to 100 c.c. of 70 per cent. alcohol.) The differentiation is controlled under a microscope and very pretty preparations of amœbæ and cysts obtained. Of course the alcoholic solution must not be allowed to act long enough to wash all eosin out of the nuclei.

1153. Methylen blue and eosin (CHENZINSKY'S formula, p. 185) sometimes stains tissues, and especially blood, exquisitely, but Trypanosomes only show blue nuclei and granules (MINCHIN, *Quart. Journ. Micr. Sci.*, liii, 1909, p. 785).

1154. MALLORY'S **eosin and methylen blue** stain, recommended for sections which have been fixed in Zenker's fluid. § 342.

A trace of colophonium should be present in the alcohol used for differentiation in order to obtain the best results.

1155. Giemsa's Stain (see §§ 875, 877). The preparation supplied by Gurr, London, is quite satisfactory.

1. For dried smears fixed with osmic vapour and absolute alcohol. Dilute each drop of stain with 1 c.c. of neutral * distilled water and place it in a clock-glass. Transfer slide from absolute alcohol and place it, smear downwards, in the stain for twenty to thirty minutes. Wash in distilled water, then tap-water, then again in distilled, and allow to dry. Red blood corpuscles should be bluish-mauve (they will be pink if fixed only in absolute, and if too much osmic acid has been used they will be blue or greenish-blue). Parasites should be blue, with red nuclei and flagella.

These dried smears are sometimes useful to compare with films made by the better wet methods, especially when very small flagellates, hæmamœbæ, etc., are being studied.

2. For wet films or sections. MINCHIN obtained excellent results with sections of fleas' stomachs fixed in Maier's modification of Schaudinn's fluid.

After washing, to remove all trace of fixative, transfer from water to stain, diluted as above, for one hour. Then leave overnight or for some hours in a weaker stain—1 drop to 4 or 5 c.c. water—rinse in water, and differentiate in acetone mixed with different proportions of xylol, beginning with 95 per cent. acetone for a very short time and ending with pure xylol.

The mounting medium must be quite neutral.

1156. The other Romanowsky stains (see § 878) are not suitable for exact protozoological work, though Leishmann's stain is useful for staining blood for diagnostic purposes, and if parasites are scarce it may be necessary to make a thick film (Ross's method) and dehæmoglobinise it in distilled water before fixing and staining (Carleton, *op. cit.*, 1926, p. 347).

* The distilled water should be stored over soda-lime or some other substance to prevent the absorption of CO_2, or it may be neutralised by Giemsa's method with K_2CO_3, using hæmatoxylin as an indicator. A 1 per cent. solution of the potassium carbonate is added drop by drop to a measured volume of the distilled water containing a few drops of a weak hæmatoxylin solution until the colour changes, after well shaking, from yellowish-red to reddish-purple. In this way the number of drops of the carbonate solution required to neutralise a given volume of the distilled water is known.

1157. Mallory's **triple stain** is useful for differentiating tissues containing parasites. Mallory (*Pathol. Technique*, 1918, p. 112) fixes in Zenker's fluid before using this stain, but Carleton (*op. cit.*, 1926, p. 132) obtains excellent results after his sublimate-formol, and says that material fixed in Bouin can be improved by placing the sections for about a minute in a saturated aqueous solution of sublimate and then washing it out well before proceeding with the stain. He differentiates the mixture as well as the fuchsin in tap-water, since all these stains can be washed out in dilute alkalies. Mallory (1918, p. 112) stains sections in 0·5 per cent. aqueous solution of acid fuchsin for a few minutes (two to four), then transfers to the following solution for ten to twenty minutes or longer :

Anilin blue soluble in water (Grübler) .	0·5 grm.
Orange G (Grübler)	2·0 ,,
1 per cent. aqueous solution of phosphomolybdic acid	100 c.c.

The sections are then washed and differentiated in tap-water, dehydrated rapidly and mounted.

Collagen fibrils, reticulum of connective tissue, mucus, chitin, etc., stain blue ; nuclei, cytoplasm, shades of red ; hæmatids, yellow to orange. Sharp (*Univ. Calif. Pub. Zool.*, xiii, 1914, p. 58) with a slightly modified method for ciliates obtains the ectoplasm mauve, endoplasm pink, macronucleus orange-brown, micronucleus and neuromotor fibres bright red.

1158. Modification of **Claudius' method** (*Annal. Inst. Past.*, xi, 1897, p. 332) of carrying out Gram's stain (§ 373). Although this is not strictly speaking a stain for protozoa, it is often most useful for staining Gram positive organisms such as yeasts, *e.g. Histoplasma, Cryptococcus*, etc., in tissues, and preventing them from being mistaken for protozoa. The following is satisfactory for films or sections after good fixation :

Stain in paracarmine or borax carmine, see §§ 271, 273.

(There is no advantage in using Orth's alcoholic carmine as recommended by Claudius—it tends to macerate and staining is no better than with the usual carmine mixtures.)

After washing the preparations, transfer them from distilled water to 1 per cent. aqueous solution of methyl violet (or carbol gentian violet) for one to two minutes.

Rinse in water and treat for one to two minutes with half-saturated aqueous solution of picric acid.

Remove as much of this solution as possible by blotting round the sections with filter paper. Do not let the preparations dry, however, and decolourise by pouring on a few drops of chloroform and then covering them with clove oil until no more blue colour comes away. They will then appear pinkish and may be washed in xylol and mounted in balsam.

Carmine does not usually stain paraffin sections easily (LANGERON, *Précis de Microscopic*, 1913, p. 369), and material such as human liver, containing *Histoplasma capsulatum*, or salivary duct of mule infected with *Cryptococcus farcinimosus* (fixed in formalin) sometimes proves impossible to stain with carmine. For sections of these I have obtained better results by staining with safranin (a saturated solution in distilled water for ten minutes) instead of carmine. It tends to wash out during the differentiation with clove oil, but after the excess of blue colour has been removed differentiation can be stopped by clearing in xylol and the nuclei left a brick-red colour, as well as any connective tissue fibres and yolk present, while the parasites (Gram positive) alone are blue.

TESTS FOR CHROMATIN

1159. Methyl green in presence of an acid is useful for staining chromatin in fresh unfixed tissues (see Chapter XVII., p. 178).

For sections fixed in sublimate, the same stain contained in the Ehrlich-Biondi-Heidenhain mixture may be used for chromatin (§ 322).

This method is purely a staining process and is not a very definite one either. It is often very difficult with granules, especially small cytoplasmic ones, to determine whether or not they acquire a greenish tint.

1160. FEULGEN'S **Reaction—a Microchemical Test for Chromatin** See Chapter XXVII. and WOODCOCK (*Journ. Roy. Army Med. Corps*, May, 1926, p. 1); ROBERTSON (*Parasit.*, xix, 1927, p. 375). Strictly speaking this is a test for thymonucleic acid, a constituent of chromatin of animal cells. It consists in hydrolysing certain purin-bodies contained in this acid, and breaking them down into groups of aldehyde nature. The test therefore consists of two parts: (1) hydrolysis, (2) the application of Schiff's reaction (fuchsin-sulphurous acid) for the presence of aldehydes. See § 623.

By this reaction any chromatin (containing thymonucleic acid) should have taken on a pink to violet tint, which is remarkably permanent and resistant, and the cytoplasm should be colourless.

Yeast cells do not give the reaction, because their chromatin contains a pentosenucleic acid, not thymonucleic acid. The same appears to be true of many plant nuclei.

WOODCOCK has also had negative results with *Sarcocystis* and the spores of *Glugea lophii*, but the microsporidian *Thelohania sp.* gave the reaction, and we have obtained it with *Thelohania mulleri* spores from *Gammarus*.

Resting nuclei of Gregarines sometimes give no reaction.

An interesting observation made by ROBERTSON is that the

kinetonucleus (parabasal body) of Trypanosomes and *Bodo* gives the reaction as definitely as the ordinary trophonucleus of these flagellates.

1161. Mitochondria and Golgi Apparatus. See special methods, Chapter XXX.

FAURÉ-FREMIET (*Arch. d'Anat. micr.*, xi, 1910, p. 485) has studied them in many protozoa, HIRSCHLER (*Anat. Anz.*, xlvii, 1914–15, p. 302) in a Gregarine, GATENBY and KING (*Quart. Journ. Micr., Sci.* lxvii, 1923, p. 381) in a Coccidian and (*idem*, lxx, 1926, p. 217) in Opalina; also KING (*idem*, lxx, 1926, p. 147) in a Haplosporidian, HORNUNG (*idem*, lxxiii, 1930, p. 135) in *Monocystis* and CAUSEY (*Univ. Calif. Pub. Zool.*, xxviii. 1926) in *Entamœba*, *Leishmania*, Ciliates and *Euglena*.

1162. Glycogen in protozoal cysts, etc., may be well stained by Best's carmine (§ 670) after fixing in Carnoy's mixture. See also under " Iodine."

1163. Chitin stains pale blue with picro-nigrosin, bright blue with Mann's and Mallory's stains. For chemical tests, see § 1189; for methods of softening, §§ 1183 *et seq.*; for penetration by fixatives § 1182.

1164. Fats (§ 648), microchemical reactions (§§ 666 *et seq*). Ultra-centrifuge (§ 732).

1165. Flagella of protozoa are well shown by overstaining in iron hæmatoxylin after any good fixation, when they will be black, or by counterstaining deeply with eosin after any hæmatoxylin nucleic stain. In smears fixed in osmic vapour and absolute alcohol, then stained with Giemsa's mixture, the flagella will be red and rather thicker than normal owing to the flattening that takes place during drying.

No special rules can be given for fixing and staining protozoa of different classes, nearly related forms often requiring different treatment; in fact, as MINCHIN points out (*Quart. Journ. Micr. Sci.*, liii, 1909, p. 786), every kind " requires its own special technique, which must be established empirically by trial, and can be discovered only to very limited extent and with great uncertainty by analogy."

CHAPTER XLV

METHODS FOR VARIOUS INVERTEBRATES

1166. RECENT methods for various invertebrates consist largely in the application of the techniques given in Chapter XXX, § 679 to § 738. This applies especially to such organisms as sponges, small chætopods, tunicates, cœlenterates, etc., and larvæ. Improvements in imbedding and cutting will be found in § 124 to § 222. These will be of special assistance with arthropods.

1167. Tunicata. A method of LO BIANCO * for killing simple Ascidians in an extended state has been given, § 24. Some forms, such as *Clavellina*, *Perophora*, *Phallusia*, *Molgula*, *Cynthia*, etc., should first be narcotised by treatment for from three to twelve hours with chloral hydrate (1 : 1000 in sea-water), then killed in a mixture containing chromic acid of 1 per cent. 10 parts, acetic acid of 50 per cent. 100 parts, and finally hardened in 1 per cent. chromic acid.

The compound Ascidians with contractile zooids may be left in clean sea-water till the zooids have become fully extended, then fixed by VAN BENEDEN's acetic acid process, § 89 (steel instruments being avoided for manipulating them). We strongly recommend this process.

S. LO BIANCO recommends for this group the chloral hydrate process, followed by fixation with sublimate or chromo-acetic acid.

CAULLERY (*Bull. Sc. France Belg.*, xxvii, 1895, p. 5) first stupefies the animals with cocaine (LAHILLE, a few drops of 5 per cent. solution to 30 c.c. of sea-water), then fixes in liquid of Flemming or acetic acid.

Most small *pelagic* Tunicates are very easily fixed with osmic acid or acid sublimate solution.

Lee found the acetic acid process very good for *Pyrosoma*. LO BIANCO puts them for a quarter of an hour into 50 per cent. alcohol containing 5 per cent. of hydrochloric acid, then into successive alcohols, beginning with 60 per cent. He kills the hard forms of *Salpa* with acetic acid of 10 per cent., the semi-hard ones with 1 per cent. chromic acid containing 5 per cent. acetic acid, the soft ones with 1 per cent. chromic acid containing $\frac{1}{50}$ per cent. osmic acid, or 10 parts of 1 per cent. chromic acid,

* References to methods of LO BIANCO in this chapter are all to his paper in *Mitth. Zool. Stat. Neapel*, ix, 1890, p. 435.

with 1 of formol and 9 of sea-water, Doliolidæ with sublimate, or the above osmic mixture, or a mixture of 10 parts 10 per cent. solution of sulphate of copper with 1 part concentrated sublimate solution, or the formol mixture.

MOLLUSCOIDA

1168. Bryozoa. For some methods of killing and fixing see §§ 10 and 27. S. Lo Bianco employs for *Pedicellina* and *Loxosoma* the chloral hydrate method, fixing with sublimate. For *Flustra, Cellepora, Bugula, Zoobothrium,* he employs the alcohol method of Eisig, § 17. For *Cristetella* see §§ 11, 19. See also Braun.

Conser (*Trans. Amer. Mic. Soc.*, xvii, 1896, p. 310) kills the fresh-water forms with cocaine, puts them for an hour into 1 per cent. chromic acid, and passes through water into alcohol, etc.

Similarly Calvet (*Hist. Nat. Bryozoaires,* Montpellier, 1900, p. 15), for marine forms.

Zschiesche (*Zool. Jahrb.*, xxviii, 1909, p. 6) fixes larvæ of *Alcyonidium* (settled down on a layer of celloidin or paraffin) with 8 parts of sublimate and 2 of acetic acid to 90 of sea-water, for twenty-five to thirty minutes.

1169. Brachiopoda. Lo Bianco kills small animals in 70 per cent. alcohol, larger ones being first narcotised with alcohol and sea-water.

Blochmann (*Untersuch. fein. Bau Brachiopoden,* Jena, 1892, p. 5) fixes principally with sublimate, macerates by the Hertwigs' method, § 568, decalcifies with 1 per cent. chromic acid (for thick shells add a little hydrochloric or nitric acid), or with nitric acid in alcohol of 50 to 70 per cent., and imbeds in paraffin or celloidin.

See also Ekman, *Zeit. wiss. Zool.*, lxii, 1896, p. 172.

MOLLUSCA

1170. Fixation. To kill Mollusca extended for dissection make up stock solution A: 90 parts absolute alcohol, 10 parts turpentine; 10 per cent. of A to 90 per cent. water. Leave in twelve to twenty-four hours. Lo Bianco narcotises Lamellibranchs for six to ten hours or more with alcohol, § 17, and then kills them.

List (*Fauna Flora Golf. Neapel,* xxvii, 1902, p. 292) narcotises Mytilidæ with 2 per cent. of cocaine in sea-water, and (for preservation of cilia) fixes in sea-water, with 10 per cent. of formol.

Lo Bianco advises that Prosobranchiata, and, amongst the Heteropoda, Atlantidæ, be narcotised with 70 per cent. alcohol,

§ 17. For Opisthobranchiata Lee recommended sudden killing with liquid of Perényi, or the acetic method, § 89. *Aplysia* may first be narcotised by subcutaneous injection of about 1 c.c. of a 5 to 10 per cent. solution of hydrochlorate of cocaine (Robert, *Bull. Scient. de la France,* etc., 1890, p. 449; *Zeit. wiss. mik.*, ix, 1892, p. 216). For Lo Bianco's various methods see the original, p. 467.

For Pteropoda in general, liquid of Perényi. *Creseis* is a difficult form. Lo Bianco advises the alcohol method, § 17. For the Gymnosomata he narcotises with 0·1 per cent. chloral hydrate.

For terrestrial Gastropods see §§ 23 and 25. Marchi (*Arch. mik. Anat.*, 1867, p. 204) gets rid of the mucus of the integument of Limax, which may be an obstacle to preparation, by putting the living animal into moderately concentrated salt solution, in which it throws off its mucus and dies in a few hours.

Lang (*Anat. Hefte,* 1902, p. 84) puts *Helix* into water with enough chromic acid to make it of a Rhine-wine colour, with an air-tight cover to the vessel, and when the animals are extruded injects into them a quarter to a half of a syringe of 1 per cent. cocaine, and after five to fifteen minutes dissects and fixes.

Heymans (*Bull. Acad. Belg.*, xxxii, 1896, p. 578) injects ethyl bromide under the skin of Cephalopoda.

Lo Bianco uses for fixing them his chromo-acetic acid, No. 1 (§ 44), with a double quantity of acetic acid, for twenty-four hours.

1171. Liver of Mollusca. Enriques (*Mitth. Zool. Stat. Neapel,* xv, 1901, p. 289) fixes the liver of *Octopus and Sepia* with sublimate. For *Aplysia* (especially in summer) alcohol, formol, and chromic mixtures are counter-indicated, on account of the carbohydrates in the cell. Sublimate is best.

1172. Nervous System of Pulmonata. B. de Nabias (*Act. Soc. Linn. Bordeaux,* 1894; *Rech. Hist. centres nerveux des Gastéropodes,* 1894, p. 23) opens the animals and fixes the ganglia for one hour in a mixture of 6 parts glacial acetic acid to 100 of 90 per cent. alcohol, or for fifteen to twenty minutes in 5 per cent. sublimate with 5 per cent. acetic acid. He stains in bulk, with Renaut's hæmatoxylic eosin, or R. Heidenhain's hæmatoxylin, or a copper hæmatoxylin of Viallanes, and imbeds in paraffin. He also stains by the rapid method of Golgi, imbedding, however, the ganglia in celloidin directly after the hardening in osmic acid and bichromate, and treating the sections with the silver (p. 500). He stains with methylen blue by treating the ganglia *in situ* for twelve to twenty-four hours with a 1 per cent. solution.

Dreyer (*Zeit. wiss. Zool.*, xcvi, 1910, p. 380) narcotises Nudibranchs with cocaine, and for studying the nerves fixes them

with MAYER's picro-formol, puts for a week into a mixture of 1 grm. of iron alum with 2 c.c. of formol and 40 of water, makes sections and stains with iron hæmatoxylin.

See also, for nerve-cells, McCLURE, *Zool. Jahrb.*, 1898, p. 17 (MANN's methyl blue and eosin, or BENDA's safranin and Licht-grün), and LEGENDRE, *Arch. mic. Anat.*, x, 1909, p. 312.

1173. Eyes of Gastropoda (FLEMMING, *Arch. mik. Anat.*, 1870, p. 441). To obtain the excision of an exserted eye, make a rapid cut at the base of the peduncle, and throw the organ into very dilute chromic acid, or 4 per cent. bichromate; after a short time it will evaginate, and remain as completely erect as if alive. Harden in 1 per cent. osmic acid, in alcohol, or in bichromate.

SMITH (*Bull. Mus. Comp. Zool. Harvard*, xlviii, 1906, p. 238) macerates eyes for at least two days in 9 parts of water with 1 of weak mixture of Flemming, followed by glycerin of 10 per cent. He bleaches them (in sections) with nitric acid and chlorate of potash.

1174. Eyes of Cephalopoda and Heteropoda (GRENACHER, *Abh. naturf. Ges. Halle-a.-S.*, Bd. xvi, 1896, p. 213). Depigment with hydrochloric acid (in preference to nitric acid). The mixture § 611 may also be used. If you stain with borax carmine and wash out in this mixture, the pigment will be found to be removed quicker than the stain is washed out.

LENHOSSÉK (*Zeit. wiss. Zool.*, lviii, 1894, p. 636; *Arch. mik. Anat.*, xlvii, 1896, p. 45) applies the method of GOLGI to the eyes of Cephalopods.

Similarly KOPSCH (*Anat. Anz.*, xi, 1895, p. 362), but using formol instead of the osmic acid.

HESSE (*Zeit. wiss. Zool.*, lxviii, 1900, p. 418) fixes eyes of Heteropoda with 1 of formol to 4 of water, and (p. 257) bleaches those of Cephalopoda by the methods of GRENACHER and that of JANDER, § 617.

See also MERTON, *ibid.*, lxxix, 1905, p. 326.

1175. Eyes of Lamellibranchiata. See PATTEN, *Mitth. Zool. Stat. Neapel*, vi, 1886, p. 733, and RAWITZ, *Jena. Zeit. Naturw.*, xxii, 1888, p. 115, and xxiv, 1890, p. 579 (bleaches with caustic soda): see § 619. HESSE (*op. cit.*, last §) employs the method of JANDER for *Arca*. He fixes the eye of *Pecten* in 10 per cent. formol for five minutes, followed by sublimate or picro-nitric acid.

1176. Shell. Sections of non-decalcified shell are easily obtained by the usual methods of grinding, or, which is often a better plan, by the methods of v. KOCK or EHRENBAUM. MOSELEY (*Quart. Journ. Mic. Sci.* (2), xxv, 1885, p. 40) decalcifies with nitric acid of 3 to 4 per cent. and then makes sections. This method serves for the study of the eyes of CHITONIDÆ.

1177. Injection of Acephala (Flemming, *Arch. mik. Anat.*, 1878, p. 252). To kill the animals freeze them in a salt and ice mixture, and throw them for half an hour into lukewarm water. They will be found dead, and the injection-pipe may be tied in the heart, and the entire animal filled and covered up with plaster of Paris, which serves to occlude cut vessels that it is not possible to tie. As soon as the plaster has hardened the injection may be proceeded with. See also Dewitz, *Anleit. zur Anfert. zootom. Präp.*, Berlin, 1886, p. 44 (*Anodonta*) and p. 52 (*Helix*).

Dakin (*Liverpool Mar. Biol. Comm.*, xvii, 1909, p. 76) narcotises by adding alcohol and glycerin for eighteen to twenty-four hours, puts for half an hour into formol of 5 per cent., and injects into a branchial vessel.

Mozejko (*Zeit. wiss. Mik.*, xxvi, 1909, p. 353, and 1910, p. 542) puts for half an hour into water at 40° to 50° C., removes the shell, and injects carmine through the heart. For occluding vessels he takes cotton-wool soaked with gelatin and plaster of Paris. He takes for a vaso-dilator a saturated solution of *peptonum siccum.*

1178. Maceration Methods for Epithelium. Engelmann (*Pflüger's Arch.*, xxiii, 1880, p. 505) macerates the intestine of *Cyclas* in osmic acid of 0·2 per cent. (after having warmed the animal for a short time to 45° to 50° C.), or in concentrated boracic acid solution.

Cilia. The entire intra-cellular fibre apparatus may be *isolated* by teasing fresh epithelium from the intestine of a Lamellibranch (*e.g.*, *Anodonta*) in either bichromate of potash of 4 per cent. or salt solution of 10 per cent. To get good views of the apparatus *in situ* in the body of the cell, macerate for not more than an hour in concentrated solution of boracic or salicylic acid. Very dilute osmic acid (*e.g.*, 0·1 per cent.) gives also good results. The "lateral cells" of the gills are best treated with strong boracic acid solution (5 parts cold saturated aqueous solution to 1 part water).

Dr. Orton uses borax carmine and picro-nigrosin (*in litera*).

Bela Haller's **Mixture,** see § 574; Brock's **Medium,** § 565; Mobius's **Media,** § 569; the second of these is much recommended by Drost (*Morphol. Jahrb.*, xii, 1866, p. 163) for *Cardium* and *Mya.*

Patten (*Mitth. Zool. Stat. Neapel,* vi, 1886, p. 736) takes sulphuric acid, 40 drops to 50 grm. of water. Entire molluscs, without the shell, may be kept in it for months.

Bernard (*Ann. Sci. Nat.*, ix, 1890, p. 191) macerates the mantle of Prosobranchs in a mixture of 1 part each of glycerin and acetic acid, 2 parts each of 90 per cent. alcohol and 0·1 per cent. chromic acid and 40 parts water, which acts in from a quarter of an hour to three hours. He also (pp. 102, 306) uses

a weak solution of chloride of ruthenium, especially for nerve-tracts, mucus-cells and cilia. Alcohol material may be macerated in a mixture of 1 part glycerin, 2 of acetic acid and 40 of water.

1179. Mucus Glands. RACOVITZA (*Arch. Zool. Expér.* (3), ii, 1894, p. 8) studies these in Nudibranchs (and Annelids) by killing with acetic acid, staining *in toto* with methyl green dissolved in liquid of RIPART and PETIT, and after three to six days, when only the glands show the stain, examining in mixture of equal parts of glycerin and the liquid.

ARTHROPODA

1180. General Methods for Arthropoda. As general methods for the study of chitinous structures, the methods worked out by Paul Mayer (see §§ 102 and 103) are excellent. It is, at all events, absolutely necessary, in the preparation of *entire organisms or unopened organs*, that all processes of fixation, washing and staining should be done with fluids possessing great penetrating power. Hence picric acid combinations should in general be used for fixing, and alcoholic fluids for washing and staining. *Concentrated* picro-sulphuric acid (or *picro-nitric*) is the most generally useful fixative, and 70 per cent. alcohol is the most useful strength for washing out. *Alcoholic* picro-sulphuric acid may be indicated for fixing in some cases.

But if the animals or organs can first be properly opened, the usual methods may be employed.

1181. Crustacea. Some forms are very satisfactorily fixed with sublimate. Such are the Copepoda and the larvæ of Decapoda. It is sometimes indicated to use the sublimate in *alcoholic* solution. Some Copepoda, however (*Copilia*, *Sapphirina*), are better preserved by means of weak osmic acid, and so are the Ostracoda. In many cases the osmic acid will produce a sufficient differentiation of the tissues, so that further staining may be dispensed with; so far *Copilia* and *Phyllosoma*. The pyrogallic process (§ 413) may be useful. GIESBRECHT takes for marine Copepods a concentrated solution of picric acid in sea-water, to which a little osmic and acetic acid may be added. For fresh-water forms, ZACHARIAS (*Zool. Anz.*, xxii, 1899, p. 72) takes chromo-acetic acid.

GIESBRECHT fixes larvæ of Stomatopoda for five to ten minutes in formol of 10 per cent. warmed to 10° or 50° C., opens them in sea-water and puts for one and a half to two and a half hours into formol 1 part and sea-water 5 parts, and brings into alcohol of 70 per cent.

STAPPERS (*La Cellule*, xxv, 1909, p. 356) fixes Sympoda in GILSON's copper formol, § 120, or in HORNELL's mixture of 100 parts of 5 per cent. formol with 40 of alcohol; and for softening

the chitin puts for twelve to thirty-six hours into 3 per cent. solution of sublimate with 5 per cent. of nitric acid.

Nettovitch (*Arb. z. Inst. Wien*, xiii, 1900, p. 3) fixes *Argulus* with liquid of Tellyesniczky, § 57, warmed to 50° C.

For Fischel's intra-vitam staining of Cladocera with alizarin, etc., see last ed.

1182. Insecta, etc. The microtomy of the Arthropoda is always tiresome, but usually very difficult in the case of the insects. The development of a very intractable type of chitin makes the sectioning of the larval, pupal or adult stages a difficult business, and the embryonic stages are almost invariably provided with masses of hard yolk.

In fixation, mixtures containing nitric acid are indicated, in imbedding celloidin, or the carbon bisulphide paraffin wax methods are the best, and in sectioning the sliding microtome, in which the knife acts with a slicing motion, is absolutely necessary.

Many years ago, we were shown a method for dealing with insect material by Professor Sir Edward B. Poulton. This consisted neither of special fixing nor imbedding methods, nor of the use of special microtomes : the object imbedded in wax, say, a hard egg, was exposed carefully on the side on which lay the yolk, and the latter was scooped out with a suitably fine instrument, under the dissecting microscope. Alternatively, the chitinous exo-skeleton of a hard ant or beetle was picked off with needles, and the object re-imbedded. It is wonderful what can be done by this method, but of course it is not applicable to every object and does not get rid of internal chitin. Great help may be got by killing beetles and such insects just after emergence before the chitin has properly hardened.

We have sectioned many types of insect eggs, and always after imbedding merely in hard wax, using the celloidin paint method, and a very sharp knife on a sliding microtome. Even moth eggs can be sectioned successfully thus.

In all cases every egg, before imbedding, and preferably while in 70 per cent. alcohol, should be pricked with a very sharp and very fine needle. This facilitates the penetration of the various reagents, and shortens time of imbedding. Heat is to be avoided whenever possible, so that if wax is to be used, the objects should be transferred to carbon bisulphide from alcohol, thence to carbon bisulphide and wax, and left overnight in an open dish in a warm place, and imbedded quickly next day in pure wax. We are often doubtful if the double celloidin wax imbedding method really helps for such objects. But it is certain that the celloidin paint method, while sectioning, is advantageous.

Fixatives which are particularly good for insects are Carnoy, Petrunkewitsch, alcoholic and ordinary Bouin, and picro-nitric.

KENYON (*Tufts Coll. Stud.*, No. 4, 1896, p. 80) fixes Pauropoda in Carnoy's acetic alcohol and chloroform, § 90, cuts them in two for staining, etc., and imbeds in celloidin followed by paraffin.

HENNINGS (*Zeit. wiss. Mik.*, xvii, 1900, p. 311) takes—Nitric acid 16 parts, chromic acid of 0·5 per cent. 16 parts, sublimate saturated in 60 per cent. alcohol 24 parts, picric acid saturated in water 12, and absolute alcohol 42, fixes for twelve to twenty-four hours, and washes out with iodine alcohol. He says that this mixture not only fixes, but softens chitin enough to allow of paraffin sections being made through hard parts.

HAMANN (*Sitz. Naturw. Freunde Berlin*, 1897, p. 2) fixes small Tracheata in 10 per cent. formol and finds the chitin sufficiently soft for sections to be made.

VAN LEEUWEN (*Zool. Anz.*, xxxii, 1907, p. 318) takes for larvæ of Hexapoda 12 parts of 1 per cent. solution of picric acid in absolute alcohol, 2 of chloroform, 2 of formol, and 1 of acetic acid.

HOLLANDE (*Arch. d'Anat. mic.*, xiii, 1911, p. 171) takes 12 parts of saturated solution of picric acid in formol of 40 per cent., 54 of absolute alcohol, 3 of benzene, and 1 of nitric acid, and finds that this fixes quickly enough not to make chitin too hard.

NUTTALL, COOPER and ROBINSON (*Parasitology*, 1908, i, p. 163) fix for a few minutes in hot picro-sulphuric acid.

1183. Methods for Clearing and Softening Chitin. The methods of Loos have been described § 587, those of HENNINGS and HAMANN last §.

In recent years the tendency has been to attack the problem of chitin as follows: (*a*) To avoid higher strengths of ethyl alcohol, substituting instead one of the other alcohols, see § 124. (*b*) To do the whole imbedding in dioxan, § 130. (*c*) To make the wax very hard by adding ceresin, § 177. (*d*) To use the proprietary substance diaphanol, § 1184. Such insects as aphids can be cut easily by avoiding ethyl alcohol and xylol, as made possible by *a* and *b*, while insects more thickly chitinised can be treated in fresh or properly kept diaphanol. See also remarks on hardness in tissues in §§ 171, 172.

1184. Diaphanol is best used on animal and plant tissues in glass-stoppered bottles at room temperature in diffused daylight.

The fixed and well-hardened tissues are rinsed in 63 per cent. alcohol and then placed in diaphanol till they are perfectly bleached and softened. In case of discoloration of the diaphanol the process must be repeated. The tissues are now placed directly in 63 per cent. alcohol. After the tissues have been well hardened in alcohol, they are then transferred through tetralin into paraffin. It is essential to pierce or cut the objects in different places prior to all procedures. All hardened objects produce CO_2, which must be given an avenue of escape.

Tetralin which is recommended for clearing diaphanol specimens is tetra-hydro-naphthalene, an imperfectly saturated hydrocarbon liquid, used in commerce as a solvent of resins, etc. It is quite possible, but not so good, to use instead benzol, or the

methyl benzoate celloidin method of Peterfi, § 177. The main point is that the diaphanol softens chitin, and in the subsequent treatment of the specimens it is necessary to avoid too much heat. In using the diaphanol method it is necessary to see that the liquid is freshly bought, or that it has been kept in a cool dark place, otherwise it does not work.

Diaphanol is a trade name for a mixed substance produced by passing the vapours of chlorine dioxide (ClO_2) into ice cold 50 per cent. acetic acid. It is not advisable to try and make diaphanol in a biological laboratory. It is obtainable from Messrs. Leitz, who supply a leaflet.

(Refer also to E. Schmidt and E. Graumann, *Ber. deutsch. chem. Ges.*, liv, 1860; P. Schulze, *Sitz. Ges. nat. Freunde*, Hefte 8/10, 1921, and *Biol. Central*, 1922; F. Duysen, *Ber. deutsch. botan. Ges.*, xcii, 1922; R. Potonie, *Die Braunkohle*, 1922.)

1185. List (*Zeit. wiss. Mik.*, 1886, p. 212) treats Coccidæ (after hardening) for eighteen to twenty-four hours with *eau de Javelle*, diluted with 4 volumes of water. After washing out they may be imbedded in paraffin, and good sections obtained.

Saling (*Dissert. Marburg.*, 1906, p. 11) boils larvæ of *Tenebrio* for some minutes in *eau de Labarraque*, the heat serving to fix the soft parts, which in successful cases are well preserved. Wash out with warm water, then alcohol.

Sazepin's method for antennæ of Chilognatha (*Mem. Acad. Imp. St. Pétersb.*, xxxii, 1884, pp. 11, 12) consists in steeping antennæ (that have been dehydrated with alcohol) for twenty-four hours in chloroform containing a drop of fuming nitric acid (shake occasionally).

Bethe (*Zool. Jahrb.*, viii, 1895, p. 544) puts *telsons of Mysis* for eight to fourteen days into 40 per cent. alcohol, to which nitric acid is gradually added, so that by the end of that time they have been brought into alcohol containing 20 per cent. of the acid. This softens the chitin, and somewhat breaks down the structure of the otolith, so that good sections through it are occasionally obtained.

Similarly Herbst, *Arch. Entwickelungsmech.*, ix, 1899, p. 291.

See also the depigmentation processes, §§ 611 to 620.

1186. Double Imbedding of Insects. A. E. Boycott dissolves 1·5 grm. desiccated celloidin chips in 50 c.c. clove oil, or, better, adds the celloidin in an ordinary ether-alcohol solution and evaporates off the solvent in the oven overnight.

It takes many days to dissolve thoroughly, but the time may be shortened by keeping it at 90° F. Fix objects in absolute alcohol; bring them into clove oil, and allow this to clear the preparations, then transfer to the clove oil celloidin. The time in this must be gauged according to the size and nature of the insect; *fleas*, if a rupture is made in the chitinous covering, are penetrated in twenty-four hours or less. When ready to imbed, dip a cover-glass in melted paraffin wax, to get a smooth surface on which the celloidin solution will not spread, but forms a *thick* drop. Place the insect on the prepared glass slip in a drop

sufficient to completely cover it, and arrange in any desired position. Invert the cover-glass and float on to some chloroform; leave for half an hour or longer, according to the size of the drop. The drop of celloidin should fall away from the glass. Transfer to melted wax, and allow time for the wax thoroughly to permeate (twenty minutes is long enough for fleas and lice). The result will be a small tablet-shaped mass of spongy celloidin impregnated with wax; this can be at once imbedded, or may be put away for future use. (Professor Boycott informs us that he has never left his preparations for more than two or three weeks at a time, so that he has no data as to how long the same could be kept, but they could probably be stored indefinitely.)

1187. Carbon Bisulphide Imbedding of Insects, etc. HEIDENHAIN many years ago recommended carbon bisulphide as a medium for imbedding in wax. The wax is dissolved in carbon bisulphide, and dehydrated insects, cleared in carbon bisulphide, etc., are placed in some of the fluid, which is allowed to evaporate at a gentle warmth. Subsequently the material is rapidly treated in pure wax in the thermostat. This method certainly curtails the length of time in the thermostat, and overheating is a serious matter when one is working at chitinous or brittle organisms

1188. Techniques for Small Arthropods. Dr. A. D. Imms informs us that for mounting and clearing aphids and other small insects, etc., the following formula as used by Professor Berlese for Acarina gives good results :—

H_2O	20 c.c.
Chloral hydrate	up to 160 grm.
Gum arabic *	15 ,,
Glucose syrup	10 c.c.
Acetic acid	5 ,,

The living specimens may be placed direct on the medium on the slide, or may be killed by a short immersion in 10 per cent. acetic acid, or boiling water. If the specimens are in alcohol they should be washed in 10 per cent. acetic acid before mounting. After the cover-glass is put on, gently warm the slide, then allow to cool, and leave for one or two weeks to dry and set. Ring the slide with a waterproof substance and finally ring with a layer of Canada balsam.

Gatenby kills in 90 per cent. of absolute alcohol, leaves for a few days, and mounts in Euparal (see § 488).

Gum Chloral. A mountant for insects, etc., used in British and other museums. We do not know original source.

Distilled water	50 c.c.
Gum arabic	40 grm.
Glycerin	20 c.c.
Chloral hydrate	50 grm.

* Dissolve the gum in the water first, add the glucose syrup, then chloral hydrate to saturation. Note that this mountant contains no glycerin (see § 455).

Dissolve the gum in the water, cold, then add the chloral and dissolve with gentle heat, then add the glycerin and filter *through cambric in a hot funnel.*

Insect Larvæ. To pass alcohol specimens to glycerin, "H. J. F.," in WATSON'S *Microscope Record*, No. 9, 1926, suggests the following ingenious method : Obtain a phial 1 inch in diameter. Pour in glycerin 1 inch in height. Take a strip of $\frac{1}{4}$ inch gummed paper and place it outside so that its lower edge is level with the surface of glycerin. Float on to the glycerin a mixture of 9 parts of 90 per cent. alcohol, 1 part ether, until its surface is level with the upper edge of the paper. Transfer the object into the upper layer with a camel-hair pencil. Evaporation should be complete in twenty-four hours, when the specimen is ready, mount in glycerin or jelly.

The beautiful specimens sold by dealers in microscope slides, and mounted in glycerin jelly, etc., are made as follows, patience being the main requisite. Kill in weak formalin ($2\frac{1}{2}$ per cent.) and bring into 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 18, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100 per cent. glycerin, leaving for a day in each solution, the first eight solutions being made with $2\frac{1}{2}$ per cent. formalin, the rest with plain water. (See D. S. SPENCE, WATSON'S *Microscope Record*, No. 25, 1932.)

For cementing glycerin preparations see § 500.

1189. Test for Chitin (ZANDER, *Pflüger's Arch.*, lxvi, 1897, p. 545). Treat for a short time with a drop of freshly prepared solution of iodine in iodide of potassium and add a drop of concentrated chloride of zinc. This is then removed with water as far as possible, and the violet reaction is obtained.

P. SCHULZE (*Sitz. Ges. natur. Freunde*, Hefte 8/10, cxxxv, 1921) uses Diaphanol (§ 1184). If pigmented, thoroughly bleach in diaphanol for at least twenty-four hours or longer. Divide the material into two parts, and soak in water. Treat one part as above (Zander), whereupon chitin turns violet. To distinguish cellulose and tunicin from chitin treat the other part with iodine and then add concentrated sulphuric acid. Chitin turns brown, cellulose and tunicin blue.

See also WEBSTER, *Zool. Jahrb., Abth. Syst.*, xxvii, 1910, p. 531.

BETHE'S **Stain for Chitin** (*loc. cit.*, § 1185). Sections are put for three or four minutes into a freshly prepared 10 per cent. solution of anilin hydrochloride, to which has been added 1 drop of hydrochloric acid for every 10 c.c. They are then rinsed in water, and the slide is put with the sections downwards into 10 per cent. solution of bichromate of potash. The stain is at first green, but becomes blue in tap-water or alcohol containing ammonia.

MAYER simply uses a solution of pyrogallol in alcohol or glycerin; and HOFMANN (*Zeit. wiss. Zool.*, lxxxix, 1908, p. 684) puts for a day or more into raw pyroligneous acid.

Dr. Orton writes to us that he simply uses picro-nigrosin and borax carmine.

1190. Tracheæ may be studied by the Golgi bichromate and silver process. Martin (*C. R. Soc. Philomath.*, 1893, p. 3) injects them with *indigo white* (through the body cavity), and puts into hot water from which the air has been expelled by boiling. Tracheæ blue.

1191. Brain of Bees. Kenyon (*Journ. Comp. Neurol.*, vi, 1896, p. 137; *Journ. Roy. Mic. Soc.*, 1897, p. 80) treats by the Golgi process (seldom successful), or hardens in a mixture of 1 part formol and 2 of 5 per cent. sulphate of copper, followed by staining in Mallory's phosphomolybdic hæmatoxylin.

Jonescu (*Jena. Zeit.*, xlv, 1909, p. 111) has employed the silver methods of Ramón y Cajal and Bielschowsky and Wolff.

1192. Ventral Cord. Floyd (*Mark. Anniv.*, 1904, p. 355) fixes the ganglia of *Periplaneta* for eighty minutes with vapour of formol, and brings into alcohol.

See also Binet, *Journ. Anat. Phys.*, xxx, 1894, p. 469.

1193. Eyes of Arthropods. For the methods of Lankester and Bourne (*Quart. Journ. Mic. Sci.*, 1883, p. 180: *Limulus*); Hickson (*ibid.*, 1885, p. 243: *Musca*); Parker (*Bull. Mus. Harvard Coll.*, xx, 1890, p. 1; *Zeit. wiss. Mik.*, viii, 1891, p. 82: *Homarus*) see *early editions.*

Parker (*Mitth. Zool. Stat. Neapel*, xii, 189, p. 1) also applies the methylen blue method to the retina and optic ganglia in Decapods, especially in *Astacus*. He injects 0·1 c.c. of a 0·2 per cent. solution into the ventral sinus. After twelve to fifteen hours the animals are killed, the ganglia quickly dissected out, and the stain fixed as described, § 382.

For his method for eyes of *Scorpions* see § 618.

For the methods of Purcell for the eyes of *Phalangida* see *Zeit. wiss. Zool.*, lviii, 1894, p. 1. He has the following stain. The cephalothorax is removed and brought for twenty minutes into 50 per cent. alcohol warmed to 45° or 50° C., and saturated with picric acid. The pigment dissolves in this solution and stains the nuclei and some other parts of the rhabdoms, so that no further stain is required.

Hennings (*Zeit. wiss. Mik.*, xvii, 1900, p. 326) depigments sections by putting them for ten minutes (*Musca*) to twelve hours (Myriopoda) into a mixture of 2 parts of 80 per cent. alcohol with 1 of glycerin and 2 per cent. of nitric acid, best kept at 35° C. The elements are well preserved.

Widmann (*Zeit. wiss. Zool.*, xc, 1908, p. 260) makes the lens of Arachnida fit for sectioning by putting for a day or so into alcohol with 10 to 15 per cent. of nitric acid; and bleaches sections with 1 part of chlorine water to 2 of alcohol.

See also ROSENSTADT, *Arch. mik. Anat.*, xlvii, 1896, p. 478; VIALLANES, *Ann. Sci. Nat.*, xiii, 1892, p. 354; and DIETRICH, *Zeit. wiss. Zool.*, xcii, 1909, p. 465 (fixes in alcoholic formol, and bleaches with dilute *aqua regia*).

1194. Ixodidæ, etc. The examination of ticks is unusually difficult, not only on account of their exoskeleton, but also because of variable degrees of distension with blood. It is best to strip off the chitin after a few hours' preliminary fixation in formalin or Regaud's fluid, which makes the underlying tissues firmer and prevents needless injury. Sometimes it is better to delay until dehydration has been commenced. Prolonged action of alcohol makes the chitin more than ever brittle. Another method with nymphs or adults engorged with blood is to squeeze the viscera out through a small opening in the exoskeleton. A pair of artery clamps is better than ordinary forceps, because with them the pressure can be regulated and maintained. With a clean, wet needle, the viscera can be freed as a single drop which keeps its shape as it falls in the fixative. For this purpose Regaud's fluid gives poor results as compared with Zenker's fluid, Giemsa's sublimate, and sublimate acetic, because in it the tissue tends to disperse instead of retaining its spherical shape. For staining, Giemsa's method is suggested (Cowdry, *Journ. Exp. Med.*, xlii, 1925, 257). For the technique of dissecting ticks see Wolbach (*Journ. Med. Res.*, xli, 1919, 67). Useful suggestions for the recognition of Rickettsiæ, Giemsa artifact and insect granules in the tissues of blood-feeding arthropods are given by Hertig and Wolbach (*Journ. Med. Res.*, xliv, 1924, 333).

VERMES

1195. Chætopoda: Cleansing Intestine. KÜKENTHAL (*Journ. Roy. Mic. Soc.*, 1888, p. 1044) puts *Lumbricus* into a glass vessel filled with bits of moistened blotting paper. They gradually evacuate the earthy particles from the gut, and fill it instead with paper.

VOGT and YUNG (*Traité d'Anat. Comp. Prat.*, v) recommend coffee-grounds instead of paper, as they cut better after imbedding.

JOEST (*Arch. Entwicklungsmech.*, v, 1897, p. 425) simply keeps the worms for a few days in moist linen, and finds the gut empty.

PEARL (*Journ. appl. Mic.*, iii, 1901, p. 680) injects alcohol of 6 per cent. through the gut of narcotised worms.

1196. Chætopoda: Fixation. *Lumbricus* may be anæsthetised by putting the animals into water with a few drops of chloroform. PERRIER puts them into water in a shallow dish, sets up a watch-glass with chloroform in the corner of it, and covers the whole.

CERFONTAINE (*Arch. de Biol.*, x, 1890, p. 327) injects interstitially about 2 c.c. of a 1 : 500 solution of curare.

JAQUET (*Bib. Anat.*, iii, 1895, p. 32) kills *Lumbricus* in extension in 1 part of nitric acid to 125 of water.

COLLIN (*Zeit. wiss. Zool.*, xlvi, 1888, p. 474) puts *Criodrilus lacuum* into a closed vessel with a little water, and hangs up in it a strip of blotting paper soaked in chloroform. KÜKENTHAL

(*Die mik. Technik*, 1885; *Zeit. wiss. Mik.*, 1886, p. 61) puts Annelids into a glass cylinder filled with water to the height of 10 cm., and then pours 70 per cent. alcohol to a depth of 1 to 2 cm. on to the water. For Opheliadæ he also employs 0·1 per cent. of chloral hydrate in sea-water.

Many marine Chætopoda may be successfully narcotised (Lo Bianco) in sea-water containing 5 per cent. of alcohol, or by means of the mixture, § 19.

The *Polychæta sedentaria* may sometimes be satisfactorily fixed by bringing them rapidly into corrosive sublimate. Cold not hot, solutions should be taken, as heat frequently shrivels up the branchiæ. *Eunice* and *Onuphis* may be treated in the same way.

Lo Bianco advises killing Chætopteridæ, Sternaspidæ, *Spirographis*, *Protula*, by putting them for half an hour into 1 per cent. chromic acid. Some of the *sedentaria* may be got protruded from their tubes by leaving them for some hours in 0·1 per cent. chloral hydrate in sea-water.

For Eisig's methods for Capitellidæ see *Fauna u. Flora Golf. Neapel*, xvi, 1887, p. 295.

See also § 12 (lemon juice), and the methods §§ 10 to 27.

1197. Blood-vessels of Annelids (Kükenthal, *Zeit. wiss. Mik.*, 1886, p. 61.) The animals should be laid open and put for two or three hours into *aqua regia* (4 parts of nitric acid to 2 of hydrochloric acid). Vessels black, on a yellow ground.

Bergh (*Anat. Hefte*, xlv, 1900, p. 392, and xlix, 1900, p. 599) puts small Annelids for a week or more into equal parts of 1 per cent. nitric acid and 1 per cent. nitrate of silver, or into 50 parts of nitrate, 25 of formic acid, and 25 of water, dissects out the organs and exposes to light. Marine forms may be treated by Harmer's process.

1198. Nerves of Annelids. Note the methylen blue method and the bichromate of silver method of Golgi (the *rapid* method). For the latter see v. Lenhossék (*Arch. mik. Anat.*, xxxix, p. 102).

Langdon (*Journ. Comp. Neur.*, x, 1900, p. 4) injects strong solution of methylen blue into the body cavity of *Nereis*, and puts the animal for some hours into sea-water in the dark, fixes the stain by Bethe's method, and makes paraffin sections.

See also M. Lewis, *Abat. Anz.*, xii, 1896, p. 292; Atheson, *ibid.*, xvi, 1899, p. 497; and the methods of Apáthy, §§ 381 *et seq.*

1199. Hirudinea. For the methods of killing see those given for *Lumbricus* in § 1195, also §§ 13 to 26.

Whitman (*Meth. in mic. Anat.*, p. 27) recommends that they be killed with sublimate.

Lee has obtained better results by narcotising with carbonic

acid (§ 25), and fixing with liquid of Flemming. He has also found that *lemon juice* kills them in a state of very fair extension.

Apáthy succeeds with alcohol of 40 per cent.

Graf (*Jen. Zeit.*, 1893, p. 165) has obtained good results by narcotising with a decoction of tobacco.

1200. Injection. Whitman (*Amer. Natural.*, 1886, p. 318) states that very perfect natural injections may often be obtained from leeches that have been hardened in weak chromic acid or other chromic liquid.

Jacquet (*Mitth. Zool. Stat. Neapel*, 1885, p. 298), for artificial injections, puts leeches into water with a very small quantity of chloroform, and allows them to remain a day or two in the water before injecting them.

1201. Nervous System. Impregnation with gold. Bristol (*Journ. of Morph.*, xv, 1898, p. 17) kills in formic acid of 15 to 20 per cent., puts for twenty-five minutes into 1 per cent. gold chloride, reduces in formic acid of 1 per cent. (twelve to eighteen hours), and imbeds in paraffin. See also §§ 376 *et seq.*

1202. Nephridia. Shearer (*Quart. Journ. Micr. Sci.*, lv, 1910, p. 288) stains *Histriobdella* intra-vitam with a very weak solution of *methyl* blue, which allows the course of the nephridia to be made out.

1203. Gephyrea. Vogt and Yung (*Anat. Comp. Prat.*, p. 373) direct that *Siphunculus nudus* be kept for some days in perfectly clean basins of sea-water, changed every day, in order that the intestines of the animals may be got free from sand, and then anæsthetised with chloroform.

Ward (*Bull. Mus. Comp. Zool., Cambridge, Harvard Coll.*, xxi, p. 144) puts them into a shallow dish with sea-water and pours 5 per cent. alcohol in a thin film on to the surface of the water, and as soon as they make no contractions on being stimulated removes to 50 per cent. alcohol.

Lo Bianco says killing with 0·5 per cent. chromic acid or with 0·1 per cent. chloral hydrate in sea-water may be tried. *Phascolosoma* and *Phoronis* should be treated by the alcohol method, larvæ of *Sipunculus* with cocaine, § 20.

Apel (*Zeit wiss. Zool.*, xlii, 1885, p. 461) puts *Priapulus* and *Halicryptus* into a vessel with sea-water and heats on a water bath to 40° C.; or they may be thrown into boiling water, which paralyses them so that they can be quickly cut open and thrown into $\frac{1}{3}$ per cent. chromic acid or picro-sulphuric acid.

1204. Rotatoria. For quieting them for study in the living state, Weber (*Arch. de Biol.*, viii, 4, 1888, p. 713) finds that 2 per cent. solution of hydrochlorate of cocaine gives the best results. Warm water gave him good results for large species, such as those of *Hydatina* and *Brachionus*.

Hardy (*Journ. Roy. Mic. Soc.*, 1889, p. 475) recommends

thick syrup added drop by drop to the water. HUDSON (*ibid.*, p. 476) mentions weak solution of salicylic acid.

VOLK (*Jahrb. Hamburg. wiss. Anst.*, xviii, 1901, p. 164) quiets them in quince mucilage, 40 grm. of the seeds to 1 litre of water.

HIRSCHFELDER (*Zeit. wiss. Zool.*, xcvi, 1910, p. 211) studies them living in neutral red of 1 : 50,000.

See also §§ 13 and 26. Methylen blue, § 376, may be found useful.

Permanent preparations may be made by the method of ROUSSELET (*Journ. Quekett Mic. Club*, v, March, 1895, p. 1): The animals are got together in a watch-glass and are narcotised by adding to the water at intervals a few drops of the mixture given in § 13.

As soon as the cilia have ceased to beat, or are seen to be on the point of ceasing to beat, they are fixed by adding a drop of liquid of Flemming or of ¼ per cent. osmic acid. After half a minute or less the animals are taken out with a pipette, and thoroughly washed by passing them through two or three watch-glasses of distilled water. They are then definitely mounted in a mixture of formol 2½ parts, distilled water 37½ parts.

ZOGRAF (*Comptes Rend.*, cxxiv, 1897, p. 245) narcotises as ROUSSELET, but without the spirit, fixes with osmic acid for two to four minutes, then replaces this by raw pyroligneous acid diluted with 8 to 10 volumes of water, and after five to ten minutes washes in several changes of water, and passes through successive alcohols into glycerin or balsam.

LENSSEN (*La Cellule*, xiv, 1898, p. 428) for the embryology of *Hydatina*, kills with hot saturated sublimate, dehydrates, stains lightly, imbeds in paraffin and stains with hæmalum.

HIRSCHFELDER (*op. cit.*, *supra*) narcotises with cocaine, and fixes with Fol's picro-chromic acid.

BEAUCHAMP (*Arch. Zool. Expér.*, iv, 1906, p. 29) finds 1 per cent. stovaine better than cocaine for some forms. He (*ibid.*, x, 1909, p. 77) fixes for five to ten minutes in 4 parts of 1 per cent. osmic acid, with 1 of 6 per cent. sublimate, and 5 of 5 per cent. bichromate of potash, and 1 drop of acetic acid for each 2 c.c., and imbeds in celloidin, and then through chloroform in paraffin (three to ten minutes).

See also TOZER (*Journ. Roy. Micr. Soc.*, 1909, p. 24).

1205. Acanthocephali. SAEFFTIGEN (*Morph. Jahrb.*, x, 1884, p. 120) obtained the best results by killing gradually with 0·1 per cent. osmic acid; the animals placed in this contract during the first hours, but stretch out again and die fully extended. Similarly with 0·1 per cent. chromic acid; Echinorhynci live for days in it, but eventually die fully extended.

HAMANN (*Jen. Zeit.*, xxv, 1890, p. 113) has succeeded with

sublimate, and also with alcohol containing a little platinum chloride.

Kaiser (*Biblioth. Zool.*, H. vii, 1, Hälfte, 1891, p. 3) found that a saturated aqueous solution of cyanide of mercury, warmed to 45° to 50° C., and allowed to act from fifteen to sixty minutes, and then washed out with 70 per cent. alcohol, was the best of all fixing media.

1206. Nematodes. The impermeable cuticle is a great obstacle to preparation. According to Looss (*Zool. Anz.*, 1885, p. 318) this difficulty may be overcome in the manner described in § 587.

Wash in 1 per cent. saline (if necessary) and fix in boiling 70 per cent. alcohol; store in fresh 70 per cent. for examination. If this method is properly applied the worms will die extended and straight. For examination transfer to 70 per cent. alcohol made up with 5 per cent. glycerin. Place small bottle of this fluid, with worms, on incubator at 60° C., and allow to evaporate slowly for about twenty-four hours or even two days, which finally leaves the worms in viscid, almost pure, glycerin. Examine in pure glycerin, or glycerin jelly. For rapid examination after killing in alcohol, transfer to absolute alcohol for thirty minutes, and clear in " white " creosote. (Leiper, in *Science of the Sea*, London. John Murray. 1912).

For fixing, most recent authors recommend sublimate solutions; chromic solutions seem to have a tendency to make the worms brittle.

But, according to Zur Strassen (*Zeit. wiss. Zool.*, liv, p. 655), *Bradynema rigidum* ought to be fixed for at least twelve hours in mixture of Flemming.

Augstein (*Arch. Naturg.*, lx, 1894, p. 255) takes for *Strongylus filaria* Mayer's picro-nitric acid.

Vejdovsky (*Zeit. wiss. Zool.*, lvii, 1894, p. 645) advises for *Gordius* 0·5 per cent. chromic acid (twenty-four hours).

Lo Bianco employs for marine forms concentrated sublimate or picro-sulphuric acid.

Looss (*Zoöl. Anz.*, xxiv, 1901, p. 309) prefers hot (80° to 90° C.) alcohol of 70 per cent.

Glaue (*Zeit. wiss. Zool.*, xcv, 1910, p. 554) kills *Ascaris* in a hot mixture of 100 parts of saturated sublimate, 100 of alcohol. and 1 of acetic acid.

Staining is frequently difficult, and sometimes alcoholic carmine § 271, is the only thing that will give fair results.

For thin sections, S. Doubrow and J. Rousset (*Bull. d'hist. appl.*, 1929) fix in mecuric chloride saturated in 80 per cent. alcohol, with 5 per cent. acetic acid for from four to twelve hours. Transfer to iodised alcohol for a few days, upgrade to 100 per cent. alcohol, then butyl alcohol for twenty-four hours. Place

in paraffin oven at 56° C. in butyl alcohol saturated with paraffin for twenty-four hours, then pure paraffin for twenty-four hours.

Braun (see *Journ. Roy. Mic. Soc.*, 1885, p. 897) recommends that small unstained Nematodes be mounted in a mixture of 20 parts gelatin, 100 parts glycerin, 120 parts water, and 2 parts carbolic acid, which is melted at the moment of using. Canada balsam, curiously enough, is said to sometimes make Nematodes opaque.

Demonstration of living Trichinæ. Barnes (*Amer. Mon. mik. Journ.*, xiv, 1893, p. 104) digests trichinised muscle (of the size of a pea) in a mixture of 3 gr. of pepsin, 2 dr. of water, and 2 minims of hydrochloric acid, kept at body temperature for about three hours. The flesh and cysts being dissolved, the fluid is poured into a conical glass, and allowed to settle; the trichinæ are drawn off from the bottom with a pipette, got on to a slide with water and examined on a hot stage.

Graham (*Arch. mik. Anat.*, l, 1897, p. 216) isolates Trichinæ by macerating for one or two days in 2 per cent. acetic acid, staining with aceto-carmine, and teasing.

1207. Nemertina. Lee's best results have always been obtained by fixing with cold saturated sublimate solution, acidified with acetic acid. The other usual fixing agents, such as the osmic and chromic mixtures, seem to act as irritants, and provoke such violent muscular contractions that the whole of the tissues are crushed out of shape by them.

Professor du Plessis has suggested to Lee fixing with hot (almost boiling) water. Lee has tried it and found the animals die in extension, without vomiting their proboscides. So also Joubin, *Bull. Mus. Hist. Nat.*, 1905, p. 326.

Lee has tried Foettinger's chloral hydrate method (§ 19). His specimens died fairly extended, but vomited their proboscides. According to Lo Bianco narcotisation with a solution of 0·1 to 0·2 per cent. in sea-water for six to twelve hours is useful.

Oestergren (§ 17) recommends his ether water.

Dendy (see *Journ. Roy. Mic. Soc.*, 1893, p. 116) has succeeded with *Geonemertes* by exposing it for half a minute to the vapour of chloroform.

For staining fixed specimens *in toto* Lee found that it was well-nigh necessary to employ *alcoholic* stains. Borax carmine or Mayer's alcoholic carmine may be recommended; not so cochineal or hæmatoxylin stains, on account of the energy with which they are held by the mucin in the skin.

Sections by the paraffin method, after penetration with *oil of cedar* (chloroform will fail to penetrate sometimes after a lapse of weeks).

Bürger (*Fauna u. Flora Golf. Neapel,* xxii, 1895, p. 443) studies the nervous system, nephridia, skin, muscle and intestine by the intra-vitam methylen-blue method. He *injects* the animals with 0·5 per cent. solution in distilled water, or 0·5 per

cent. salt water, and allows them to lie for six to twelve hours or more in moist blotting paper.

See also MONTGOMERY (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 6); and BÖHMIG (*Zeit. wiss. Zool.*, lxiv, 1898, p. 484).

1208. Cestodes. Wash gently in 1 per cent. saline, and then fix in hot corrosive sublimate acetic (at *circa* 50° C.) and allow the tape-worms to remain in the dish till the fluid becomes cold. Wash in running water for twelve hours and transfer to 70 per cent. alcohol. Stain as in general methods.

As pointed out by VOGT and YUNG (*Traité d'Anat. Comp. Prat.*, p. 204), the observation of the living animal may be of service, especially in the study of the excretory system. And, as shown by PINTNER, Tæmiæ may be preserved alive for several days in common water in which a little white of egg has been added.

TOWER (*Zool. Jahrb.*, xiii, 1899, p. 363) has kept *Moniezia expansa* alive for several days in a mixture of 100 c.c. of tap-water, 10 grm. of white of egg, 2 of pepsin, 2 of sugar, and 5 of prepared beef ("Bovox"). Chloride of sodium, he says, should be avoided.

LÖNNBERG (*Centrbl. Bakteriol.*, xi, 1892, p. 89; *Journ. Roy. Mic. Soc.*, 1892, p. 281) has kept *Triænophorus nodulosus* alive for a month in a slightly acid pepsin-peptone solution containing from 3 to 4 per cent. of nutritive matter and less than 1 per cent. of NaCl.

For the nervous system, TOWER (*Zool. Anz.*, xix, 1896, p. 323) fixes in a picro-platin-osmic mixture (stronger than that of O. VOM RATH, § 106) for ten hours, then treats for several hours with crude pyroligneous acid, and lastly with alcohol, and imbeds in paraffin.

ZERNECKE (*Zool. Jahrb., Abth. Anat.*, ix, 1895, p. 92) kills *Ligula* in the osmio-bichromic mixture of GOLGI (4 : 1), impregnates as usual, makes sections in liver, and treats them by the hydroquinone process of KALLIUS. Besides the peripheral and central nervous system, muscle-fibres, parenchyma cells, and the excretory vascular system are impregnated.

He has also obtained good results by the methylen-blue method.

BLOCHMANN (*Biol. Centrbl.*, xv, 1895, p. 14) recommends the bichromate and sublimate method of GOLGI.

See also KÖHLER, *Zeit. wiss. Zool.*, lvii, 1894, p. 386 (stretches Tæniæ round a glass plate or on cork, and fixes with 5 per cent. sublimate); LÜHE, *Centrbl. Bakt.*, xxx, 1901, p. 166, and RANSOM, *U. S. Nation, Mus. Bull.*, lxix, 1909, p. 8.

1209. Trematodes. If necessary, clean by shaking up in 1 per cent. saline (parasites). Decant off dirty liquid, one-third of the

tube is filled again with 1 per cent. saline, in which the worms are shaken vigorously, and an equal quantity of $HgCl_2$ solution is added quickly, the vigorous shaking being continued for several minutes thereafter. This treatment should kill the flukes in an extended condition. Leave in the fixer as indicated (corrosive one or two days, wash in water twelve hours if 10 per cent. formalin be substituted for the $HgCl_2$; leave about same time and store in 3 per cent. formalin).

FISCHER (*Zeit. wiss. Zool.*, 1884, p. 1).—*Opisthotrema cochleare* may be mounted entire in balsam. For sectioning, he recommends a mass made by dissolving 15 parts of soap in 17·5 parts of 96 per cent. alcohol. The sections should be studied in glycerin.

LO BIANCO fixes Trematodes with hot saturated sublimate.

LOOSS (*Arch. mik. Anat.*, 1895, p. 7) takes for *Bilharzia* warm (50° to 60° C.) 1 per cent. sublimate in 70 per cent. alcohol.

BETTENDORF (*Zool. Jahrb., Abth. Morph.*, x, 1897, p. 308) has had good results with the rapid Golgi method only on *Distoma hepaticum*, and prefers methylen blue.

HAVET (*La Cellule*, xvii, 1900, p. 353) has also had results with the Golgi method on this form, and also with thionin (after fixing with sublimate), which demonstrates tigroid substance.

Cercariæ. SCHWARZE (*Zeit. wiss. Zool.*, xliii, 1886, p. 45) found that the only fixing agent that would preserve the histological detail of these forms was cold saturated sublimate solution warmed to 35° to 40° C.

For an "indifferent" liquid, HOFMANN (*Zool. Jahrb.*, xii, 1899, p. 176) takes 1 part of white of egg in 9 of normal salt solution.

1210. Turbellaria. BRAUN (*Zeit. wiss. Mik.*, iii, 1886, p. 398) gets entire animals (Rhabdocœla) on to a slide, lightly flattens out with a cover, and kills by running in a mixture of 3 parts of liquid of Lang with 1 of 1 per cent. osmic acid solution. (BÖHMIG [*ibid.*], commenting on this, says that for some of the tissues, such as muscle and body parenchyma, nitric acid and picro-sulphuric acid are very useful.) Sections may be made by the paraffin method.

DELAGE (*Arch. de Zool. exp.*, iv, 2, 1886) recommends fixation (of Rhabdocœla Acœla) by an osmium-carmine mixture, for which see *loc cit.*, or by concentrated solution of sulphate of iron. Liquid of Lang was not successful.

For staining, he recommends either the osmium-carmine or impregnation with gold ($\frac{1}{3}$ formic acid, two minutes; 1 per cent. gold chloride, ten minutes; 2 per cent. formic acid, two or three days in the dark).

BÖHMIG (*Zeit. wiss. Mik.*, iii, 1886, p. 239) has obtained instructive images with Plagiostomidæ fixed with sublimate and stained with the osmium-carmine.

GRAFF (*Turbellaria Acœla*, Leipzig, 1891; *Zeit. wiss. Mik.*, ix, 1892, p. 76) says that chromo-aceto-osmic acid, followed by hæmatoxylin, is good for the skin, but not for the Rhabdites,

which in Accœla and Alloiocœla seem to be destroyed by swelling. The same method is also good for the parenchyma of *Amphichœrus cinereus*, *Convoluta paradoxa* and *C. sordida*. Sublimate is good for *Convoluta Roscoffensis*. The nervous system may be investigated by the methods of DELAGE.

For Dendrocœla sublimate solutions, sometimes hot, appear indicated for fixing ; see the mixture of LANG, § 69, also CHICHKOFF (*Arch. de Biol.*, xii, 1892, p. 438).

ARNOLD (*Arch. Zellforsch.*, iii, 1909, p. 433) kills *Dendrocœlum* in extension with strong liquid of Flemming.

OESTERGREN narcotises *Dendrocœlum* with his ether-water, § 17. JAENICHEN (*Zeit. wiss. Zool.*, lxii, 1896, p. 256) advises for *Planaria*, eyes especially, picro-sulphuric acid for an hour or two ; osmic acid is not good, and liquid of Müller macerates. He stains with borax carmine, makes sections, and puts them for ten minutes into osmic acid, then for five minutes into pyroligneous acid, on the top of the stove. He macerates the visual rods in a mixture of 1 part common salt, 1 of acetic acid, and 100 of water. He bleaches the pigment of the eyes with peroxide of hydrogen.

WILHELMI (*ibid.*, lxxx, 1906, p. 548) throws Triclads into almost boiling mixture of Zenker, and after ten to thirty minutes removes to water for some hours, and then passes into iodine alcohol.

ECHINODERMATA

1211. Holothurioidea. These are difficult to fix on account of their contracting with such violence under the influence of irritating reagents as to expel their viscera through the oral or cloacal aperture.

VOGT and YUNG (*Anat. Comp. Prat.*, p. 641) say that *Cucumaria Planci* (*C. doliolum*, Marenzeller) is free from this vice ; but they recommend that it be killed with fresh water, or by slow intoxication, § 19.

Synapta may be allowed to die in a mixture of equal parts of sea-water and ether or chloroform (S. LO BIANCO).

OESTERGREN (§ 17) puts *Synapta* into his ether water, but Dendrochirota first into magnesium sulphate of 1 to 2 per cent., for some hours.

GEROULD (*Bull. Mus. Harvard Coll.*, xxix, 1896, p. 125) paralyses *Caudina* with sulphate of magnesia, § 23, and fixes with liquid of Perenvi (or sublimate for the ovaries).

Holothurids, Dr. WEBER informs us, are admirably preserved in formaldehyde ; a weak solution is sufficient.

For the staining of muscles with methylen blue, see IWANZOFF, *Arch. mik. Anat.*, xlix, 1897, p. 103, and for the study of calcareous plates, see WOODLAND, *Quart. Journ. Micr. Sci.*, xlix, 1906, p. 534 (fixation with osmic acid, staining with picro-carmine, followed by Lichtgrün).

1212. Echinoidea. Lee advises that they be killed by *injection* of some fixing liquid. For preservation, formaldehyde has proved *admirable* in all respects, and greatly superior to alcohol (Weber).

Lo Bianco kills by pouring over them (mouth upwards) a mixture of 10 parts acetic acid and 1 of 1 per cent. chromic acid, and brings at once into weak alcohol. Or he makes two holes in the shell, lets the water run out and alcohol in.

Sections of spines may be made by grinding, see §§ 202, 910.

Spicula and the skeleton of pedicellariæ may be cleaned by *eau de Jarvelle*, see Döderlein (*Wiss. Ergeb. Tiefsee-Exped.*, v, 1906, p. 67).

1213. Asteroidea. Hamann (*Beitr. Hist. Echinodermen*, ii, 1885, p. 2) *injects* the living animal with a fixing liquid through the tip of a ray. The ambulacral feet and the branchiæ are soon distended by the fluid, and the animal is then thrown into a quantity of the same reagent.

In order to study *the eyes*, with the pigment preserved *in situ*, they should be removed by dissection, should be hardened in a mixture of equal parts of 1 per cent. osmic acid and 1 per cent. acetic acid, and sectioned in a glycerin gum mass, or some other mass that does not necessitate treatment with alcohol (which dissolves out the pigment, leaving the pigmented cells perfectly hyaline). For maceration use one-third alcohol, the aceto-osmic mixture failing to preserve the rods of the pigmented cells.

Specimens for externals only preserve in 70 per cent. alcohol or formalin. They retain their shape better if they are put for two or three minutes into fresh water before being placed in the fixer. If the internal anatomy is to be studied, cut along the length of each arm so as to allow fluid to enter, and preserve in 2 per cent. chromic acid, etc. Wash in running water, transfer to 70 per cent. alcohol ; or the specimen may be preserved in formalin spirit or 5 per cent. formalin.

Formaldehyde is *not* to be recommended for the cell preservation of Asteroidea (Weber). See also Lo Bianco, *op. cit.* (he kills *Brisinga* with absolute alcohol), also §§ 14 *et seq.*

1214. Ophiuridea should in general be killed in *fresh water* if it be desired to avoid rupture of the rays (De Castellarnau, *La Est. Zool. du Napoles*, p. 135).

Lo Bianco kills small forms with weak alcohol, *Ophiopsila* with absolute alcohol, and *Ophiomyxa* with 0·5 per cent. chromic acid.

Russo (*Richerche Lab. Anat. Roma*, iv, 1895, p. 157) fixes *Ophiothrix* for an hour or two in 0·5 per cent. osmic acid and then decalcifies in solution of Müller for six to ten days. Or he fixes for three minutes in a mixture of 2 parts concentrated sublimate solution, 1 part 70 per cent. alcohol, and 1 part acetic acid (sp. gr. 1·06), and decalcifies in Müller or in 70 per cent.

alcohol with 10 per cent. of acetic acid. He stains with paracarmine.

1215. Crinoidea. Lo Bianco (*loc. cit.*, p. 458) fixes *Antedon rosacea* with 70 per cent. alcohol, *A. phalangium* with 90 per cent.

1216. Larvæ of Echinodermata (from instructions written down for Lee by Dr. Barrois). For the study of the metamorphoses of the Echinoidea and Ophiuridea it is necessary to obtain preparations that show the *calcareous skeleton preserved* intact (a point of considerable importance, since this skeleton frequently affords landmarks of the greatest value), and that give clear views of the region of formation of the young Echinoderm (which is generally opaque in the living larva). They should also possess sufficient stiffness to allow of the larva being turned about in any desired way, and placed in any position under the microscope.

Pluteus larvæ should be fixed in a cold saturated solution of corrosive sublimate, for not more than two or three minutes, then washed with water, and brought into dilute Mayer's cochineal (§ 249). This should be so dilute as to possess a barely perceptible tinge of colour. They should remain in it for from twelve to twenty-four hours, being carefully watched the while, and removed from it at the right moment and mounted in balsam, or, which is frequently better, in oil of cloves or cedar wood.

Auricularia and Bipinnaria. As above, but the earlier stages of the metamorphosis of Auricularia are better studied by fixing with osmic acid, staining with Beale's carmine, and mounting in glycerin.

Larvæ of Comatula are best fixed with liquid of Lang, and stained with *dilute* borax carmine. It is important (for preparations that are not destined to be sectioned) to use only *dilute* borax carmine, as the strong solution produces an overstain that cannot easily be reduced.

Narcotisation by chloral hydrate before fixing is useful, especially for the study of *Pentacrinus* larvæ and of the young *Synaptæ* formed from Auricularia. Without this precaution you generally get preparations of larvæ either shut up (*Pentacrinus*), or entirely deformed by contraction (young *Synaptæ*).

See also MacBride on the development of *Amphiura squamata*, *Quart. Journ. Micr. Sci.*, xxxiv, 1892, p. 131 (osmic acid followed by liquid of Müller and alcohol; decalcification with nitric acid in alcohol; staining with Mayer's paracarmine or hæmalum); and Seeliger on the development of *Antedon*, *Zool. Jahrb.*, *Abth. Anat.*, vi, 1892, p. 161.

MacBride (*Quart. Journ. Micr. Sci.*, xxxviii, 1896, p. 340) fixes larvæ of *Asterina* in osmic acid, brings into liquid of Müller for twelve to fourteen hours, imbeds in celloidin followed by paraffin (see § 190), and stains sections with carmalum or Delafield's hæmatoxylin, best after a foregoing stain of twenty-four hours in borax carmine.

Mayer (*Grundzüge*, Lee and Mayer, 1910, p. 486) arranges a number of fixed and stained *Plutei* on a sheet of gelatin foil

gummed to a slide with euparal, dehydrates by adding alcohol by drops, and adds euparal and a cover. See also WOODLAND, *Quart. Journ. Micr. Sci.*, xlix, 1905, p. 307.

CŒLENTERATA

1217. Thread-Cells. IWANZOFF (*Bull. Soc. Nat. Moscou*, x, 1896, p. 97), advises for the Nematocysts of Actiniæ maceration by HERTWIGS' method, § 568, or better, fixation for two to five minutes with vapour or osmium followed by a short washing with sea-water or distilled water.

For Medusæ he also advises HERTWIGS' method, *loc. cit.*, or treatment with a solution containing methyl green and gentian violet with a little osmic acid.

1218. LITTLE (*Journ. App. Mic.*, vi, 1903, p. 2116; *Journ. Roy. Mic. Soc.*, 1903, p. 237) kills *Hydra* in hot saturated sublimate in 70 per cent. alcohol, washes with alcohol, stains for five minutes in strong solution of methylen blue, dehydrates rapidly, clears with cedar or bergamot oil, and mounts in balsam. Nematocysts blue, the rest unstained.

1219. Actinida. Anæsthetise in menthol (§ 14), which will take some twelve hours or more. For ordinary sea anemones, formalin (5 to 10 per cent.) followed by formalin spirit is to be preferred. For corals, such as Caryophyllia, Alcyonium or Gorgonia, anæsthetise, and then add hot corrosive sublimate or 5 per cent. formalin, followed by cold saturated corrosive sublimate. Ninety per cent. spirit, not allowed to get weaker than 70 per cent., gives good results for anatomical work (ALLEN and BROWNE, *loc. cit.*).

JOHN BAKER'S **Methods for the Micro-anatomy of Sea Anemones** (communicated). To anæsthetise a sea anemone, place it in a finger-bowl three-quarters full of sea-water, add a pinch of menthol, place it in a dark cupboard, and leave it for about twelve hours (overnight is convenient). At the end of this period it will not be completely anæsthetised, and the addition of a fixative will be likely to cause some contraction. To avoid this, complete the anæsthesia by adding, fairly gradually, 50 or 100 c.c. of 30 per cent. magnesium chloride solution. (If the magnesium chloride is used without the previous treatment with menthol, the animal will generally contract.) Leave for an hour, and then squirt the fixative with a pipette all round the animal, and, if possible, into the throat. Then pour away the sea-water and substitute the fixative. A convenient fixative is Heidenhain's "Susa." Dealcoholisation may be done in cedar-wood oil. The following staining method depends on the fact that after the use of a fixative (such as Susa) which contains formaldehyde, carmine is inhibited from staining all parts *other than the mesoglœa.* It is

therefore easy to show up the mesoglœa alone in red, and thus display the anatomy advantageously.

Sections may be thick or thin. Stain in borax carmine until the mesoglœa is fairly strongly stained, but everything else still scarcely tinged. This takes from half an hour to several hours. Wash in a stream of distilled water from a wash-bottle. Stain for four minutes in Mayer's acid hæmalum. Blue. (No differentiation is required.) Wash in distilled water. Dip for less than ten seconds in $\frac{1}{4}$ per cent. light green in 70 per cent. alcohol. Before the ten seconds have elapsed, wash the stain off in a stream of distilled water. Dehydrate at the ordinary speed. Mount in balsam. Mesoglœa, red. Nuclei, blue. Nematocysts, green.

For other *narcotisation* methods see §§ 17 to 26.

1220. *Fixation.* In *Le Attinie, Fauna u. Flora d. Golfes v. Neapel*, ANDRES says that hot corrosive sublimate often gives good results. In the case of the larger forms the solution should be injected into the gastric cavity.

Freezing sometimes gives good results. A vessel containing Actiniæ is put into a recipient containing an ice and salt freezing mixture and surrounded by cotton-wool. After freezing, the block of ice containing the animals is thawed in alcohol or some other fixing liquid.

DUERDEN (*Journ. Inst. Jamaica*, ii, 1898, p. 449) narcotises with magnesium sulphate, § 23, and fixes with formol of 3 to 5 per cent.

1221. *Maceration.* For the HERTWIGS' method (*Jen. Zeit.*, 1879, p. 457) see § 568. The tissues should be left to macerate in the acetic acid for at least a day, and may then be teased in glycerin.

LIST (*Zeit. wiss. Mik.*, iv, 1887, p. 211) treats tentacles of *Anthea cereus* and *Sagartia parasitica* for ten minutes with a mixture of 100 c.c. of sea-water with 30 c.c. of Flemming's strong liquid, then washes out for two or three hours in 0·2 per cent. acetic acid, and teases in dilute glycerin. Picro-carmine may be used for staining.

1222. *Nervous system.* This group is generally held to be refractory to the Golgi impregnation. HAVET, however (*La Cellule*, xviii, 1901, p. 388), has obtained good results by the rapid method on young specimens of *Metridium dianthus*. Besides nerve-cells, there are impregnated neuro-muscular cells, gland-cells, and nematocysts. He leaves for five to eight days in the osmic mixture. He has also had good results by the intra-vitam methylen blue method (this is also good for nematocysts). So also has GROSELJ (*Arb. Zool. Inst. Univ. Wien*, xvii, 1909, p. 269), adding the dye to the water with the animals till it gives a steel-blue tint.

1223. Zoantharia with Calcareous Skeletons are difficult to deal with on account of the great contractility of the polyps. Sublimate solution, which ought very often to be taken boiling, sometimes gives good results.

See also Lo Bianco, *loc. cit.*, p. 446.

Sections. See §§ 910 and 202, for undecalcified specimens.

1224. The Alcyonaria have also extremely contractile polyps. In a former edition Lee suggested for their fixation either hot sublimate solution or glacial acetic acid (§ 12). S. Lo Bianco has since recommended essentially similar processes. Garbini (*Manuale*, p. 151) drenches them with ether, and brings into strong alcohol.

Wilson (*Mitth. Zool. Stat. Neapel*, 1884, p. 3) kills Alcyonaria with a mixture of 1 part of strong acetic acid and 2 parts of concentrated solution of corrosive sublimate, the animals being removed as soon as dead and hardened for two or three hours in concentrated sublimate solution.

1225. Zoantharia and Alcyonaria. Braun (*Zool. Anz.*, 1886, p. 458) inundates *Alcyonium palmatum*, *Sympodium coralloides*, *Gorgonia verrucosa*, *Caryophyllia cyathus*, and *Palythoa axinellæ* with a mixture of 20 to 25 c.c. of concentrated solution of sublimate in sea-water with 4 to 5 drops of 1 per cent. osmic acid, and after five minutes passes into successive alcohols.

(This method also gives good results with *Hydra* and some Bryozoa and Rotifers.)

See also §§ 14 *et seq.*

Bujor (*Arch. Zool. Expér.*, ix, 1901, p. 50) kills *Veretillum* in sea-water containing 10 per cent. each of formol and ether, and after a minute passes into 2 per cent. solution of formol in sea-water.

1226. Hydroidea in General. Directly the tow-net comes on board, the Plankton must be poured into a glass jar and jelly fishes at once picked out by means of a lifter or pipette, and placed in another very clean jar of sea-water. Leave in this jar for half an hour to allow organisms to recover from shock. Note that the slightest trace of chemicals in the jar will prevent their expanding. The secret of successful preservation depends on keeping the animals in motion while you pour in the fixer. First stir the organisms very slowly and gently, and when all are in motion begin to pour the formalin slowly down the side of the vessel. About 10 c.c. of 10 per cent. formalin should go to 100 c.c. of sea-water, but better more than this quantity. Keep stirring for at least two minutes after addition of fixer. Leave for a few hours and then transfer to 5 per cent. formalin; finally store in 10 per cent. To obtain medusæ in a nice state of expansion it is necessary to use an anæsthetic (see especially §§ 16 *et seq.*). Hydrochloride of cocaine is possibly the best; use a 1 or 2 per

cent. solution. Place the medusæ in a small glass vessel with just enough sea-water to allow them to swim. After they have expanded add a little cocaine (3 c.c. of 1 per cent. solution for every 100 c.c. of sea-water). If the medusæ at the end of ten to fifteen minutes do not contract when touched with a glass-rod no more cocaine is needed; if they are still active add more narcotiser and stir; an overdose will cause prolonged contraction. After anæsthetising add the formalin and keep stirring, and continue for a minute, or longer. Do not leave specimens in solutions of cocaine longer than necessary. (ALLEN and BROWNE in *Science of the Sea*. London. John Murray. 1912.)

For further description of *narcotisation* methods see §§ 16 *et seq.*

For killing by *heat* see § 11.

Fixation. In general polyps may be very well killed in saturated sublimate solution, in which they should be plunged for an instant merely, and be brought into alcohol. The solution should be employed cold in general for Gymnoblastea, hot for most Calyptoblastea.

Ether attentively administered gives good results with Campanularidæ. *Hydra* is very easily killed by a drop of osmic acid on a slide.

For the *methylen blue intra-vitam* method, see Chapter XVIII.; also HADZI, *Arb. Zool. Inst. Wien*, xvii, 1909, p. 225.

1227. Medusæ: Fixation. For narcotisation see § 16 and above.

Trachymedusæ and Acalephæ may be fixed in the usual way in chromic or osmic mixtures. Osmic acid may be added to the sea-water containing the animals, which should be removed to spring water as soon as they begin to turn brown.

BIGELOW (*Mem. Boston Soc. nat. Hist.*, v, 1900, p. 193) fixes the scyphistomes of *Cassiopeia* in Lo Bianco's mixture of 10 parts of 10 per cent. solution of cupric sulphate with 1 of saturated sublimate, and hardens them in 5 per cent. bichromate of potash.

Medusæ: Sections. Paraffin and collodion are certainly not satisfactory as all-round methods for these watery organisms. The HERTWIGS (*Nervensystem der Medusen*, 1878, p. 5) imbedded in liver with the aid of glycerin gum, and hardened the objects and the mass in alcohol.

See also JOLIET's glycerin gum method, and the gelatin methods on page 97.

Medusæ: Maceration. See, especially for the study of the nervous system, § 568. Doubtless in many cases the pyrogallic acid reaction, § 413, would give enhanced differentiation.

1228. Siphonophora. For the cupric sulphate method of BEDOT (*Arch. Sci. phys. et nat.*, xxi, 1889, p. 556), which is

admirable for the preparation of museum specimens, but not necessary for histological work, as well as for those of Lo Bianco (*op. cit.*, p. 454), Friedlander (*Biol. Centrbl.*, x, 1890, p. 483), and Davidoff (*Anat. Anz.*, xi, 1896, p. 505) see *previous editions.* Lo Bianco fixes most forms with sublimate in sea-water.

For preserving, according to Weber, formaldehyde is better than alcohol. Davidoff (*loc. cit.*) fixes in it.

1229. Ctenophora : Fixation. *Never store in formalin,* always in 70 per cent. alcohol. *Pleurobrachia* are best killed in 5 per cent. formalin in sea-water. Fill large measuring jar with this fluid, drop in the animals and leave till they sink to the bottom; transfer to 5 per cent. formalin in pure water. After a week or so (not longer) transfer to very dilute alcohol, and upgrade to 70 per cent. strength. *Beroe :* Bring into small quantity of sea-water, and when expanded add large quantity of corrosive sublimate saturated solution in sea-water. When specimens become white, decant, and add fresh water ; wash in several changes to remove corrosive, upgrade to 70 per cent. alcohol. *Bolina* dissolves at once in formalin ; kill in Flemming, selecting small specimens ; leave half an hour, wash slightly, upgrade to 70 per cent. alcohol. (Allen and Browne in *Science of the Sea.* London. John Murray. 1912.)

Small forms are very easily prepared by means of osmic acid. For the large forms see Lo Bianco, *loc. cit.*, p. 457. He uses his copper sulphate mixture, § 1227.

Samassa makes sections by the double-imbedding method. See *Arch. mik. Anat.*, xl, 1892, p. 157.

1230. Plankton, Preservation of, without Sorting (E. J. Allen and E. T. Browne in *Science of the Sea.* John Murray. 1912). Preservation of whole catch of a tow-net is performed by stirring around the Plankton with a rod and adding a little 5 to 10 per cent. formalin. Keep on stirring for about a minute, then allow the organisms to settle to the bottom ; as soon as this occurs pour off as much of the liquid as possible and transfer the Plankton to a bottle ; again allow to settle and reduce the fluid to a minimum then fill the bottle with 5 or 10 per cent. formalin. A bottle should be not more than half full of Plankton. After a few days, or on the appearance of opalescence of the fluid, change the liquid. Another method is first to fill the Plankton by pouring some saturated solution of picric acid into the jar containing the organisms, then add some formalin 5 or 10 per cent. and leave for an hour or two, occasionally stirring. Finally decant and add 5 or 10 per cent. formalin as before described ; the yellow colour of the fluid can be neglected. Never use corrosive sublimate with formalin, as crystals form, which adhere to the organisms.

PORIFERA

1231. Spongiæ: Fixation. The smaller forms can be fairly well fixed by the usual reagents, osmic acid being one of the best. For the larger forms absolute alcohol is apparently the best. If any watery fluid be preferred, care should at all events be taken to get the sponges into strong alcohol as soon as possible after fixation, on account of the rapidity with which maceration sets in in watery fluids. FIEDLER (*Zeit. wiss. Zool.*, xlvii, 1888, p. 87) has been using (for *Spongilla*), besides absolute alcohol, an alcoholic sublimate solution and the liquids of Kleinenberg and Flemming.

1232. Staining. To avoid maceration, Lee held that alcoholic stains should be alone employed, and recommended Mayer's tincture of cochineal, § 249. VON LENDENFELD (*Zeit. wiss. Mik.*, xi, 1894, p. 22) uses aqueous solutions of Congo red and anilin blue for the coloration of collar-cells.

MINCHIN (*Quart. Journ. Mic. Sci.*, xl, 1898, p. 569) stains spicula sheaths with Freeborn's picro-nigrosin, p. 353.

ROUSSEAU (*Ann. Soc. Belg. Mic.*, xxiv, 1899, p. 51) stains in nigrosin, picro-nigrosin, or indulin, or MAYER's picro-magnesia carmine.

Prof. Dendy informed us that he used Hickson's brazilin (§ 417) a great deal in his work on sponges.

For intra-vitam staining, see LIOSEL, see last ed.

For silvering, see § 387.

Sectioning. Calcareous sponges may be decalcified in alcohol, acidified with hydrochloric or nitric acid, and then imbedded in the usual way. Siliceous sponges may be desilicified, § 610.

For ROUSSEAU's methods, see § 610. VOSMAER and PEKELHARING decalcify with a solution of picric acid in absolute alcohol (see *Zeit. wiss. Mik.*, xv, 1899, p. 462).

See also Johnstone-Lavis and Vosmaer, § 204.

Preparation of Hard Parts. Siliceous spicules are easily cleaned by treating them on a slide with hot concentrated nitric or hydrochloric acid, or solution of potash or soda. The acids mentioned are very efficient, but may attack the silex of some delicate spicules. Potash solution is, therefore, frequently to be preferred, notwithstanding that, in my experience, it does not give such clean preparations.

According to NOLL, *eau de Javelle* is preferable to any of these reagents, see § 612.

Embryos and Larvæ. MAAS (*Zool. Jahrb., Abth. Morph.*, vii, 1894, p. 334) fixes larvæ in liquid of Flemming or Hermann one to three minutes, and stains with borax carmine, or with gentian violet and orange G (Flemming). He also (*Zeit. wiss.*

Zool., lxvii, 1900, p. 218) fixes young Sycons in absolute alcohol and stains with ammonia carmine (spicules *in situ*).

Delage (*Arch. Zool. Expér.*, x, 1892, p. 421) fixes larvæ of *Spongilla* that have settled down on cover-glasses for three minutes in absolute alcohol, stains in alcoholic carmine, and brings through alcohol into oil of bergamot, then either mounts direct in balsam, or detaches the larvæ from the cover and imbeds in paraffin (three minutes).

Gatenby (*Journ. Linnean Soc.*, 1920) uses methods for mitochondria, especially Champy-Kull and Kopsch.

PART III

CHAPTER XLVI

BOTANICAL TECHNIQUE *

GENERAL METHODS OF FIXATION IMBEDDING SECTIONING AND MOUNTING

1233. Introductory. The fundamental methods described for animal tissues are in general equally applicable to those of plants. No special killing agents are required; the fixative fulfils this function. Plant material must be kept in first-class condition until it can be studied or fixed and preserved. Just prior to killing, portions for examination should be cut free of unwanted parts and immediately immersed in the fixative. Further trimming is best done below the surface of the liquid.

Better results usually accompany the use of more careful treatments, provided that effective reagents are used in every stage from fixation to the final mount. *No amount of later care can correct inadequate fixation.* It is only too evident that in both histological and cytological work many fallacious observations are traceable to insufficient care during the early stages of treatment of the material upon which the observations are later based. Less exacting methods are, of course, permissible providing it is clearly recognised that only limited demands can be made upon the finished product. For validity of fixation images see § 1355.

General references: Belling, *The Use of the Microscope*, 1928; Chamberlain, *Methods in Plant Histology*, 5th ed., 1932; Dop and Gautier, *Manuel de technique, histologie et microbie végétales*, Paris, 1928; Kisser, *Botanische Mikrotechnik*, Jena, 1926; Krause, *Enzyklopädie der mikroskopischen Technik*, 3rd ed., Berlin, 1926; Rawlins, *Phytopathological and Botanical Research Methods*, Wiley, 1933; Schneider-Zimmerman, *Die botanische Microtechnik*, Jena, 1922; Smith, *Trans. Amer. Micr. Soc.*, xxxiv, 1915, p. 71 (historical); Taylor, *General Botanical Microtechnique* (in McClung, *Microscopical Technique*, 1929).

1234. Fixatives. For rough anatomical work, in which the cell wall structure is most important, fix and preserve in 4 per cent. formalin or 70 per cent. alcohol. Carnoy and saturated alcoholic

* By D. G. C.

solutions of corrosive sublimate or picric acid give better results. For objects which easily turn black, OVERTON (*Zeit. wiss. Mik.*, vii, 1890, p. 9) uses alcohol containing sulphurous acid. An aqueous or alcoholic solution of picric acid may also be combined with sulphurous acid.

WARRINGTON (*Ann. Bot.*, xl, 1926, p. 27) uses a mixture of 3 grm. corrosive sublimate, 3 c.c. glacial acetic acid and 100 c.c. 70 per cent. alcohol, afterwards washing in 70 per cent. alcohol until iodine is no longer decolourised. Such thorough washing must follow all corrosive sublimate fixations. More delicate materials require better fixation. The following widely used fixatives are roughly in order of excellence: Flemming and its modifications (§ 1356), Navashin (§ 1357), chromo-acetic (§ 44), Bouin (§ 1358), aceto-formalin alcohol (§ 1235), formalin alcohol (§ 1235). For greater precision of action smaller pieces of material should be used; in Flemming fluids the diameter should not exceed 3 to 4 mm., and is preferably much less. No effort should be spared to expose the critical cells and tissues to the direct action of the fixative. Except with formalin, alcohol and formalin alcohol (which are also preservatives), the material should be washed well and brought gradually into 70 per cent. alcohol, in which it is stored.

See also COBB, *Trans. Am. Micr. Soc.*, xlvi, 1927, p. 153 (thermolethe for hot fixatives); GATES *Science*, xxxi, 1910, p. 234. Marine organisms should be fixed in fluids made up with sea-water; see § 1240 and § 1378.

1235. Preservatives. For general purposes, 70 per cent. alcohol is the safest; add 5 to 20 per cent. of glycerin (Calberla's fluid) to minimise the effects of evaporation. Reduce the alcohol concentration to 50 per cent. for woody or tough material, and increase to 85 per cent. for delicate material. Formalin, at 4 per cent. strength, keeps material in a less brittle state than strong alcohol. Calcified specimens should be kept in alcohol or neutral formalin. Prepare the latter by adding borax until the solution gives a red colour with phenolphthalein.

Methyl alcohol may be substituted for ethyl, but denatured alcohol should be avoided owing to the cloudiness of the aqueous solutions.

Other preservatives: 96 c.c. of 70 per cent. alcohol and 4 c.c. of formalin; 100 c.c. of 50 per cent. alcohol, 6·5 c.c. formalin and 2·5 c.c. glacial acetic acid.

See also §§ 1286 *bis*, 1378 and 1393.

1236. Preservation of Special Substances. Calcium Carbonate or Sulphate Deposits. Fix in alcohol free from acids. Cystoliths should be stained, rapidly dehydrated, cleared and mounted in balsam. A small degree of solution is difficult to avoid; cystoliths should not be left in tap-water, in which they are often soluble.

Cellulose, as food reserve in seeds, etc., is preferably collected before maturity. Cut sections under water rather than alcohol. If dry and mature, treat like hard woods.

Collenchyma and pectic substances are troublesome if too soft. Preserve and cut in 70 per cent. alcohol, swelling with water afterwards if required. A stronger alcohol may be used to harden collenchyma if necessary, but subsequent swelling is more difficult.

Crystals, as cell inclusions, show best in sections of fresh material mounted in water or in 2 per cent. acetic acid. Mounted in balsam they lose too much in visibility.

Inulin. Cut material into small blocks and treat with several changes of absolute alcohol to remove the water rapidly and completely. Cut with a knife flooded with absolute alcohol. Stain sections with a saturated solution of orange G in clove oil, wash in xylol and mount; inulin appears darker in colour than the general tissues.

Resin, *e.g.* in Gymnospermæ, is preserved and stained by immersion in saturated aqueous copper acetate solution for one to several weeks; wash the excess of copper acetate out and preserve in 50 per cent. alcohol. The resin retains a bright green colour when the sections are mounted in glycerin.

Starch is better preserved in alcohol than in formalin or acidic solutions which might hydrolyse it. Usually, however, it is preferable to be rid of it.

1237. Decalcification should be carried out as gently as possible. Dilute hydrochloric acid (2 per cent.) is most generally useful; the concentration may be increased up to 10 per cent. for more rapid results. Acetic acid is more gentle, nitric acid more violent. If the material is soft, fix in formalin alcohol, harden, and decalcify in strong alcohol by cautious addition of acetic acid. Formalin, oxidising to formic acid, will reduce or remove lime from specimens, especially if small in amount. Perényi's solution is also sometimes used. For more accurate preservation of calcified material fix in chromo-acetic, sublimate acetic, Flemming, etc., and repeatedly exhaust the material under a vacuum pump.

1238. Desilification is usually carried out by means of hydrofluoric acid, either full or half-strength, in a wax or wax-coated bottle. The chemically pure reagent should be used for more delicate material, but the commercial preparation suffices for most purposes. Dry woods should be boiled in water and exhausted of air before treatment with HF. (See also §§ 1252 and 1253.) Whole organs after fixation should be thoroughly freed of air by means of a vacuum pump while soaking in strong alcohol. If parts of the material are rather delicate, demineralisation is better carried out in weak alcohol, than in water, to avoid maceration.

Diaphanol (Leitz) is suitable for softening all indurated tissues (lignified and chitinised, as well as silicified).

1239. Herbarium material (or dried crude vegetable drugs) for anatomical study, either following imbedding and sectioning or maceration, should first be moistened with alcohol to reduce the air film and then soaked in water. Gentle heat may be applied to hasten the process, and a vacuum pump should be used to free the material from all air. Various methods have been adopted for softening and clearing such material.

LAGERHEIM (*Hedwigia*, 1888, p. 58; *Rev. Mycol.*, xi, 1889, p. 95) softens dried algæ and fungi in water and then gently warms them in concentrated lactic acid until they show small bubbles. This method is less certain with other, more resistant, material. The addition of phenol, recommended by Zimmermann, is of little help on old material, the gel colloids of the walls having lost part of their reversibility. Lacto-phenol and lacto-glycerin have also been used.

MCLEAN's method (*New Phyt.*, xv, 1916, p. 103). Place the material directly into absolute alcohol for at least twenty-four hours. Pieces of large size should also be subjected to a reduced pressure (10 cm. mercury or less) during their immersion; delicate and small objects do not require this treatment. Next, grade down very gradually to distilled water, in which the material may be left indefinitely. The growth of moulds is prevented and recovery hastened by placing in a hot chamber (*e.g.*, paraffin oven). Material in bulk soaks out better than sections. When the material is well soaked, transfer to 4 to 8 per cent. aqueous potash for six to nine days. Better results are obtained if, after half the period of immersion is over, the potash is allowed to concentrate to one-third its original volume. The potash must not be heated, otherwise the cellulose tissues would be destroyed. A reduced pressure assists removal of air and is to be employed during the concentration of the potash. Neutralise the potash with several changes of dilute acetic acid (15 to 20 per cent.); avoid the use of mineral acids. Then wash in water until the latter is neutral to litmus. If the material is still deeply coloured, the acetic acid may be shaken up first with bleaching powder, excess of which is removed by filtration.

The possibilities of the method are limited by the method of drying. The more thorough and rapid has been the drying, the better is the resuscitation.

See also ARBER, *Ann. Bot.*, xl, 1926, p. 447; *ibid.*, xliii, 1929, p. 41.

Staining presents some difficulties, since the solvent action of potash on lignin affects the reactions of lignified walls. McLean recommends a 2 per cent. aqueous solution of fuchsin or a solution of fuchsin decolourised by sulphurous acid for ten to fifteen minutes. After the latter stain, wash in tap-water until excess acid is removed. Counterstain with light green in clove oil after dehydration.

Horton (*Ann. Bot.*, xxxviii, 1924, p. 404) finds that prolonged treatment with dilute sodium hypochlorite solution brings material, particularly of flowers, into a surprisingly good condition.

1240. Dehydration. Conduct with regard to the nature of material, its bulk and the purpose (anatomical or cytological) to which it is to be put. Anatomical material of a more resistant nature, after washing, may be passed through 15, 25, 50, 70, 95 per cent. alcohols to absolute with three to six hours in each grade. More delicate material requires an extended series with 10 per cent. intervals between grades, and one to two or more hours in each. Cytological material and very delicate algal and fungal material may need closer grades, especially in the lower and upper parts of the series, viz., 2½, 5, 10, 15, 20, 25, 30, then by 10 per cent. intervals to 90, 95, and 100 per cent., with one to two hours or more in each.

According to Kisser (*Zeit. wiss. Mik.*, xlvi, 1929, p. 269), the use of absolute alcohol is unnecessary. Transfer from 95 or 96 per cent. alcohol through mixtures of alcohol and xylol to xylol; imbed in paraffin or mount in balsam. Mixtures of alcohol and benzene may be used, and in fact are preferable as water is more soluble in benzene than xylene.

Hartridge (*Journ. Physiol.*, liv, 1920, p. 8) recommends amyl alcohol, in place of absolute alcohol and clove oil, following 95 per cent. alcohol and followed by Canada balsam in xylol; and also for passing to No. 1 petrol for paraffin imbedding (*q.v.*).

Sass (*Stain Tech.*, vii, 1932, p. 65), McFarland (*Science*, lvi, 1922, p. 43), and Lyon (*Science*, lvii, 1923, p. 644), use acetone in place of alcohol.

See also Hill, *Bot. Gaz.*, lxi, 1916, p. 255 (glycerin dehydration); Bradbury, *Science*, lxxiv, 1931, p. 225 (isopropyl alcohol); Courtney, *Science*, lxvii, 1928, p. 225.

Marine plant material, fixed in solutions compounded of sea-water, are to be brought in to fresh water before dehydration. Dehyhration without shrinkage or the production of cloudiness can usually be effected by passing through the following mixtures :—

Fresh water . .	5	10	20	30	35	40	50
Sea-water . .	90	80	65	50	35	20	0
Alcohol. . .	5	10	15	20	30	40	50

and thereafter through mixtures of fresh water and alcohol only. Filamentous and unicellular organisms are often best dehydrated by placing them in 5 per cent. aqueous glycerin and allowing this to concentrate, not too rapidly (at least two days), in a place protected from dust. Later replace the concentrate with 95 per cent. or absolute alcohol. See Hemenway, *Science*, lxxii, 1930, p. 251.

Dilute (10 per cent.) aqueous phenol can also be used and

allowed to concentrate, but its action is too harsh except for clearing whole unstained mounts.

Overton's method for microscopic objects (*Zeit. wiss. Mik.*, vii, 1890, p. 9). After fixation in a drop on a slide or coverslip, add a drop of 10 to 20 per cent. alcohol and support the preparation above absolute alcohol in a close chamber. A shallow dish with its upper edge ground and sealed with a vaselined glass plate is an efficient container. The chamber must be set in an even temperature, away from insolation. In a few hours the drop becomes almost absolute. Then add a drop of very dilute alcohol-ether-celloidin solution (about one-fifth the strength of the first solution used for celloidin imbedding) and spread it evenly by tipping the preparation back and forth. As soon as the celloidin no longer appears to flow, immerse the slide in 80 per cent. alcohol, wet side up. The film becomes hard in a few minutes and the material can be further handled without fear of loss.

See also Metz, *Anat. Rec.*, xxi, 1921, p. 373; Allen, E., *Science*, lxvi, 1927, p. 427; Radir, *Science*, lxxi, 1930, p. 613.

1241. Clearing of Bulk Material. Any glycerin used in the preservative or for dehydration must first be removed by repeated changes of alcohol. An alcohol-xylol series is most used for clearing, but alcohol-chloroform seems preferable. Cedar oil is good; place some in a phial and an equal amount of absolute alcohol containing the material above it. The objects sink through into the oil and become impregnated; wash in clean oil and replace with xylol.

Hartridge uses No. 1 petrol (see § 1240).

1242. Clearing of Material for Mounting. Xylol is most used; a graded alcohol-xylol series is indicated if material tends to collapse. Clove oil is very useful, it will clear sections from 95 per cent. alcohol and is frequently used as a differentiating agent; wash out well with xylol afterwards. Anilin oil is hard on stains and cedar oil easily clouds with atmospheric moisture.

With celloidin sections a mixture of 25 per cent. or less crystallised carbolic acid and 75 per cent. or more xylol is probably best; Eycleshymer's fluid sometimes injures the stain.

Other Methods. In §§ 124, 133 are given other methods used in animal microtomy and also applicable to plant material.

1243. Imbedding. For a general account of botanical methods, see Kisser (*Abderhalden Handb. biol. Arbeitsmethoden, Abt.*, xi, Teil 4, pp. 391, and 533). The use of paraffin is advised wherever possible; that of collodion or other media only where they are imperative.

1244. Paraffin Method. See Kisser (*Cytologia*, iv, 1933, p. 288) for a critical survey. The solvents in general use are xylol and chloroform. Plant material requires more time than

animal material owing to the impedance of the cellulose walls. For this reason the addition of fragments of wax in the cold, followed by further addition when the phial (still stoppered) is on the oven top, is beneficial. Continue the additions until the solvent is saturated with wax. Then place in an open dish inside the oven to evaporate off the solvent. The times should be shortened as much as possible consistent with the avoidance of shrinkage and the distortion of the material.

Another method is to employ a series of graded xylol-paraffin mixtures, keeping the material two to six hours in each. Those below the point of saturation are used at room temperature, those above saturation need to be placed on or in the oven.

According to LAND (*Bot. Gaz.*, lix, 1915, p. 397) the usual procedure results in almost immediate surrounding of the object by a dense layer of dissolved paraffin, since both are heavier than xylol. This is not the case with the denser chloroform. He recommends filling a wire gauze support with paraffin fragments and suspending it 2 to 3 cm. above the object. Xylol sufficient to rise 1 to 2 mm. above the wax is added. The stoppered bottle is left undisturbed until saturation is reached. Thereafter treat as usual. In the case of very delicate structures, however, it is preferable to pour off the xylol-paraffin mixture repeatedly down to the support, refilling the latter each time with paraffin. See also GOODSPEED (*Bot. Gaz.*, lxvi, 1918, p. 381) for another procedure, and WEATHERWAX (*Bot. Gaz.*, lxviii, 1919, p. 305), who considers such procedures unnecessary.

Taylor recommends the following method: Half fill a fairly deep dish (1 cm. or more) with wax and allow it to set. Place the material, covered with xylol, on the wax and leave until the mass is of a soft pasty consistency, and then place in the oven. After half to one hour replace with melted paraffin wax; make two further changes at intervals of half an hour and then imbed.

DOWSON'S **Quick Paraffin Method** (*Ann. Bot.*, xxxvi, 1922, p. 577). Material after dehydration, by concentration in glycerin and its replacement with absolute alcohol, is transferred to a mixture of melted paraffin (52° C. m.p.), xylol and absolute alcohol in the proportion 1 : 2 : 3, and placed, stoppered, inside the oven. After twenty-four hours, allow the xylol and alcohol to evaporate off and imbed in fresh wax. The method is suitable for anatomical purposes.

Some material, especially that containing much starch, is often hard to cut in paraffin. *Storage of the paraffin blocks in water for several weeks softens the material and facilitates sectioning.* See also COUCH, *Science*, lxxii, 1930, p. 607.

VEH (*Ber. Deutsch. bot. Ges.*, l, 1932, p. 42) uses soft wax (m.p. 46° C.) instead of hard and cools the block to — 3° C. prior to section-

ing. This shortens the imbedding time and gives smooth sections in a continuous ribbon with a relatively dull knife.

RAU (*J. Indian Bot. Soc.*, viii, 1929, p. 131) describes the use of vacuum flasks in paraffin imbedding. See CAMPBELL, *Bot. Gaz.*, xiii, 1888, p. 158 (chloroform method).

For the ceresin wax method see § 177. Read also §§ 172 and 173.

1245. Collodion Method. Introduced into botanical technique by BUSSE (*Zeit. wiss. Mik.*, viii, 1891, p. 462). Schering's celloidin or phytoxylin, for extreme transparency, are commonly used. Photographic guncotton (JEFFREY, *Anatomy of Woody Plants*, 1926, p. 449) is satisfactory if transparency is not a desideratum.

The periods of immersion in the celloidin solutions should be lengthened for plant material. Small delicate objects (root-tips, thin textured leaves and stems) require three or four days to one week; large objects, especially blocks of wood and tissues with thick cellulose walls may require up to one month.

The methods principally in use are approximately those of Eycleshymer. It is usually best to saturate the material with ether alcohol from absolute alcohol and then transfer to 2 per cent. collodion solution in ether alcohol. Stopper the bottle loosely to allow it to concentrate gradually. Harden in chloroform.

Modifications have been described by PLOWMAN (*Bot. Gaz.*, xxxvii, 1904, p. 451) for use with hard tissues. Use ten grades of collodion, viz., 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 per cent., transferring from one to the next. Nearly fill the bottle, clamp or wire on the stopper, and put the bottle in a paraffin oven at 50–60° C. for twelve to eighteen hours. After reaching 20 per cent. concentration, add further chips of dry collodion. Blocks are cleared (and left indefinitely) in a mixture of equal parts of glycerin and 95 per cent. alcohol. See also BAILEY, *Bot. Gaz.*, xlix, 1910, p. 57, and JEFFREY, *Bot. Gaz.*, lxxxvi, 1928, p. 456.

STOCKWELL's rapid method (*Science*, lxxv, 1932, p. 291). Imbed leaf and soft stem material (of *Hedera* and *Olea*) in an acetone solution of collodion under reduced pressure; the preparation of sections, mounted in balsam on slides, requires only fifty minutes.

See also WETMORE (*Stain Tech.*, vii, 1932, p. 37) for the uses of collodion in botanical technique. Also CAROTHERS, *Science*, lxvii, 1928, p. 400 (serial sections)

1246. Double Infiltration with Collodion and Paraffin (see also § 190.) After hardening in chloroform, infiltrate with paraffin. This makes possible the cutting of ribbons of thinner sections.

REICHARDT and WETZEL (*Zeit. wiss. Mik.*, xlv, 1928, p. 476) use a modification of Peterfi's method for imbedding hard or

brittle objects. Dehydrate to absolute alcohol and clear in methyl benzoate (instead of benzol) until material is infiltrated and sinks. Treat two to five days, according to size, with 1 per cent. celloidin in methyl benzoate. Replace with methyl benzoate to which paraffin chips have been added and leave twelve to twenty-four hours at 40° C. Finally place the objects in melted paraffin at 50° C., changing this paraffin at least three times, and imbed. Avoiding benzol, chloroform and xylol prevents hardening and shrinkage.

STEHLI and KOLUMBE (*Handb. der mikr. Tech. Abt.*, xii, 1929) also give a method of imbedding in a mixture of paraffin and celloidin. See also DE ZEEUW, *Papers Mich. Acad., Sci.* i, 1923, p. 83; CHURCH, *Science*, xlvii, 1918, p. 640; KORNHAUSER, *Science*, xliv, 1916, p. 57; DAHLGREN, *J. Appl. Micr.*, i, 1898, p. 67.

1247. Gelatin methods should be used for diseased bark and wood that is likely to crumble and become brittle in paraffin or collodion imbedding and for material that would be excessively hardened in alcohol. The method of NICHOLAS (§ 180) is most suitable. Following LAND (*Bot. Gaz.*, lix, 1915, p. 400), soak gelatin in water until no more is imbibed. Drain off the excess water and liquefy the gelatin by heat. Place pieces of the material in the melted gelatin for several hours, together with small blocks of hard wood to act as supports in the microtome. Orient the material on the wooden blocks in a gelatin matrix, cool to set the gelatin and plunge into strong formalin to harden it.

See also COLLAZO, *La inclusion, en gelatina.* Montevideo, 1927; HIRINGA, *C. R. Soc. Biol.*, xci, 1924, pp. 671 and 951.

If the sections are strong enough, remove the gelatin with warm water with or without a little ammonia. Otherwise mount in glycerin jelly.

1248. Jeffrey's Glycerin Jelly Mass Method of Imbedding (*Bot. Gaz.*, lxxxvi, 1928, p. 458). The method is valuable for cutting large numbers of small objects in a predetermined plane. Dehydrate the material to strong alcohol and then transfer to equal parts alcohol and glycerin and leave overnight. Arrange the objects as desired, under the low power of a dissecting microscope, on a strip of heavy paraffin paper in a small drop of alcohol glycerin and leave it in a warm place to concentrate. Invert the strip of paraffin paper bearing the objects on to a strip of cardboard spread with melted glycerin jelly (dissolve one part of gelatin in six parts of water and then add an equal volume of glycerin). Place a small slide and a small weight on top. When the jelly has set ($\frac{1}{2}$ hour), place in 90 per cent. alcohol to harden. Later remove the paraffin paper and imbed the jelly strip in nitrocellulose. No doubt the method could be adapted to paraffin imbedding.

For other methods used in imbedding animal material see §§ 173, 181.

1249. Freezing is little used, though it can be useful for frail pathological specimens and soft or gelatinous algæ and fungi. Its use is absolutely essential in the histochemical study of the distribution of diffusible salts. Bring fixed material into 4 per cent. formalin and coat with egg albumen; gelatin or gum arabic to attach it to the carrier. Lay the sections on slides coated with gelatin and cooled. Warm the slides to make the sections adhere. Avoid stains likely to colour the gelatin. Refer to Chapter XII. See KISSER, *Zeit. wiss. Mik.*, lxv, 1928, p. 433; LIPPINCOTT, *Stain Tech.*, i, 1926, p. 39.

1250. The use of **Soap** is advocated by WILCOX (*J. Appl. Micr. and Lab. Methods*, i, 1898, p. 68), and OSTERHOUT (*Univ. Cal. Publ. in Bot.*, ii, 1904, p. 87), for material that cannot be safely dehydrated, *e.g.*, algæ, mucilaginous and other delicate structures. Saponify 70 c.c. hot coconut oil with 38·5 c.c. of 28 per cent. aqueous KOH. When firm, pulverise the product. Place material in warm water and add the soap until the solution is quite concentrated. Then dry until the mass is firm enough to attach to a wooden block in a sliding microtome. Attach sections to albumened slides, moisten with xylol and press into contact. Dissolve away the soap and warm the slide, or immerse in 96 per cent. alcohol to coagulate the alcohol.

1251. General. The use of a centrifuge to aid imbedding has been advocated by Weber. Slow centrifuging does not disturb the arrangement of the cell contents and is preferable to the use of a vacuum pump. See KISSER, *Protoplasma*, iii, 1928, p. 507.

1252. Sectioning of Woods and other Hard Objects. This requires powerful apparatus, grinding methods or a method of softening the hard tissues. Soft woods and the alburnum of harder woods can usually be cut by means of a heavily built sliding microtome. THOMSON (*Bot. Gaz.*, l, 1910, p. 148) describes a modified Jung-Thoma microtome for hard woods. See also JANE (*Ann. Bot.*, xlix, 1935, p. 398) for a device for the setting of knives.

A heavy steel plane with a blade of hard temper and a sharp straight edge is employed to obtain fairly thin large sections. The stony tissues of seeds and fruits may be sectioned by a grinding process. Cut thin sections with a fine saw and grind them down with fine, wetted carborundum powder on a piece of plate glass. At first carry out the rubbing down with the finger on the section; when the section has become quite thin (less than ½ mm.) use a piece of plate glass. When grinding is complete, wash the section, dehydrate, clear and mount in thick, warm balsam. The thin sections can be stained, but the structure usually shows well in unstained mounts.

Seeds with a reserve of hard cellulose are usually softened sufficiently by boiling in water.

1253. Demineralisation by means of hydrofluoric acid usually brings about considerable softening. This removes silica completely, has little effect upon the middle lamella and leaves the coarse contents intact. Kerr (*Trop. Woods,* xl, 1934, p. 37) states that softening by HF is associated with partial degradation of cellulose and the formation of hydrocellulose; softening is correlated with a loss of tensile strength of the cellulose. The blocks of wood for treatment should be cut to expose true transverse, radial, and tangential surfaces. Brown (*Bull. Torrey Bot. Club.*, xlvi, 1919, p. 127) suggests 2 cm. radial length, 8 mm. vertical height, and a tangential width of 6 mm. Occasional species require blocks of different, or larger, dimensions to display higher rays or other special features. The blocks should be marked with numbers for record and with arrows to indicate the direction of growth.

Boil the specimens, dry or fresh, in water and exhaust under a vacuum pump to free them of air. Brown removes the air by alternate boiling and cooling. Soak in HF (full strength or diluted) for one to several weeks, changing the liquid occasionally. When maximum softness has been attained (found by testing with a knife), wash out the acid by washing four days in running water, and soak the blocks in glycerin. Imbed if necessary; celloidin is usually recommended; adopt a rapid method if possible.

Langdon (*Bot. Gaz.*, lxx, 1920, p. 82) describes the action of HF (both pure and diluted) on lignified structures, and pays especial attention to the time of immersion of tissues in the acid. She imbeds in paraffin: dehydrate with alcohols (five grades: 60, 70, 80, 95 and 100 per cent. for twelve hours each) after soaking several days or weeks in equal parts of glycerin and 30 per cent. alcohol (Langdon, *Bot. Gaz.*, lxv, 1918, p. 313), and clear in xylol (four grades, twelve to twenty-four hours each). Remove any air or gases remaining by means of a vacuum pump when the material is in pure xylol. Infiltrate thoroughly with paraffin and section on a sliding microtome. A rotary microtome can be used if the objects are small and have much soft tissue.

Jeffrey (*Anatomy of Woody Plants*) after HF treatment soaks blocks of uniform texture in a mixture of equal parts of glycerin and 30 per cent. alcohol; those of some size or heterogeneous texture in equal parts of glycerin and 90 per cent. alcohol.

Lek (*Landbouwhogesch. Wageningen, Lab. Tuinbouwplantenteelt,* 1928 (5), p. 1) shortens the process of demineralising hard tissues to one or two days by heating for one hour in an autoclave at 16° C., using full strength HF.

Chowdhury (*Ann. Bot.*, xlviii, 1934, p. 308) shortens the process by keeping the material in HF in unstoppered gutta percha bottles, in a pressure cylinder.

1254. Jeffrey's **Vulcaniser Method** (*Bot. Gaz.*, lxxxvi, 1928, p. 456). Soften tissues in a dental vulcaniser at a temperature of about 320° F. (160° C.). The time required varies—a three to four years old oak twig needs one hour, a piece of seasoned oak four to five hours. Brass piping (¾ to 1 inch diameter) is cut into lengths to fit the vulcaniser, and the ends are threaded for brass caps. On one end the cap is made tight by sweating lead solder into the thread. The other end is made tight by putting into the cap a piece of cardboard and, on top of the cardboard, a piece of lead. Place the tube in a vice, put the water or alcohol with the material into the tube and screw the cap tight with a wrench.

After vulcanising, cool the material slowly and then treat it for a few days in a mixture of 2 parts water and 1 part hydrofluoric acid. Wash well, dehydrate and preserve in equal parts of 95 per cent. alcohol and glycerin until needed for cutting.

Jeffrey cut transverse sections of coconut shells and hard woods as thin as 2 to 3 μ.

1255. Williamson's **Cellulose Acetate Method** (*Ann. Bot.*, xxxv, 1921, p. 139). Transfer material, free of all air, from water direct to pure acetone for one to two hours and then to a 12 per cent. solution of cellulose acetate in acetone for a period depending on the hardness of the wood. The material is both softened and imbedded by this method. Soft woods require two days as a minimum, oak and beech at least six days, very hard woods about fourteen days. Staining is unaffected by the treatment.

1255 *bis*. Kisser (*Cytologia*, v, 1934, p. 520) finds that blocks $1 \times 1 \times \frac{1}{2}$ cm., kept in 96 per cent. alcohol saturated with phenol, at 60° C. over a water bath, are rapidly softened.

1256. Kisser's **Steam Method** (*Zeit. wiss. Mik.*, xliii, 1926, p. 346) consists, essentially, in allowing steam to play upon the block as the sections are being cut. A 300 c.c. conical flask half filled with water and heated from below by a Bunsen burner, is fitted with a rubber bung through which passes a thistle funnel and a bent glass tube. The thistle funnel dips well below the water level and is used for refilling the apparatus. The bent glass tube conducts steam from the flask to the block. The temperature of the steam should be about 90° C. If too hot, the steam dries the material, and if much cooler than 90° C. little advantage accrues. Kisser cut transverse sections 5 mm. square, of ebony, and sections of coconut shell 2 mm. square and 6 μ thick.

It is an advantage to boil the pieces of wood, cut into small blocks of suitable size for sectioning, for twenty-four hours. Then

put them into equal parts of 95 per cent. alcohol and glycerin for a week or more. Very hard woods may also need treatment with hydrofluoric acid; a 25 per cent. solution for a week, followed by washing and steeping in alcohol glycerin, should suffice.

EVANS and CROWELL (*Stain Tech.*, v, 1930, p. 149) have described a similar method. Water is boiled slowly in an Erlenmeyer flask from which the vapour is led to the microtome through a small copper tube in which there are a few coils. A Bunsen burner beneath the coils vaporises the water before it leaves the tube. The steam flows over the piece of wood to be sectioned. The authors have cut up to 500 sections of very hard wood with a single sharpening of the razor. They also suggest a possible improvement, which consists in using tubing of aluminium or monel metal instead of copper.

1257. LARBAUD'S **Butyl Alcohol Method** (*C. R. Acad. Sci.*, Paris, clxxii, 1921, p. 1317). Butyl alcohol softens wood and permits smooth sectioning of material that would otherwise be brittle and difficult to cut. It can be used for dehydration and clearing. See § 127.

ZIRKLE'S schedule (*Science*, lxxi, 1930, p. 103) permits more gradual dehydration than Larbaud's method.

Stage	1	2	3	4	5	6	7	8	9	10	11
Water	95	89	82	70	50	30	15	5	0	0	0
Ethyl alcohol	5	11	18	30	40	50	50	40	25	0	0
Butyl alcohol	0	0	0	0	10	20	35	55	75	100	100

Leave one hour in each stage, over night at stage 6. Leave some time in pure butyl alcohol, so that all the water is extracted. Two-thirds fill a vial with paraffin. Let the paraffin harden and place the material on it, cover the specimens with butyl alcohol and place in a paraffin oven. As the paraffin melts the material sinks and comes into contact with almost pure paraffin ; the butyl alcohol remains floating on the top. Two changes of paraffin are sufficient, the length of time in each depending upon the size of the specimens. Slight traces of butyl alcohol in the paraffin blocks do not render them crumbly, as does xylol.

Butyl alcohol does not soften wood which has been hardened by fixation, by drying or by too rapid dehydration. The method is also excellent for delicate materials.

ZIRKLE (*ibid.*) has also used PAINTER'S method (*Anat. Rec.*, xxvii, 1924, p. 77), in which anilin oil replaces the higher concentrations of alcohol. Replace the anilin oil with methyl salicylate (oil of wintergreen) and pass from the latter into paraffin.

1258. Sectioning of Cotton and other Fibres. DENHAM (*Nature*, cvii, 1921, p. 299) uses a modification of BRECKNER'S method

(*Zeit. wiss. Mik.*, xxv, 1909, p. 29). Fix the fibres in a wire frame and wet them out with alcohol. Place in a dilute syrupy solution of celloidin in alcohol ether and allow to evaporate to half volume. Then place fibres, after gently squeezing, in a chloroform solution of paraffin wax for two hours. Cut from frame, place in paraffin and quickly imbed. Section at once.

Kisser and Anderson's modification (*Amer. Journ. Bot.*, xv, 1928, p. 437) has several advantages. Very thin sections in any desired plane can be cut. Stain the fibres with carmalum and wash in water, then stretch them as much as possible and dry them between filter paper with light pressure. While stretched, cement them with gum arabic by their ends over a central window 1 cm. square, with the aid of small strips of cardboard. When the gum is dry, imbed the whole frame in celloidin and then cautiously cut out the central square of celloidin containing the fibre. Harden this in chloroform and imbed in paraffin of 52° C. melting point. To cut the suitably orientated block, wet the knife with water containing 0·1 to 0·5 per cent. gelatin or soap to reduce surface tension. Cross sections down to 2 μ and longitudinal sections down to 4 μ have been obtained. Such thin sections may be used for X-ray studies of the cell wall. Affix with albumen and remove the paraffin and celloidin with xylol and ether respectively. Make mounts in glycerin or glycerin jelly.

1259. Chemical Sectioning of Fibres. Boil the fibre in sulphuric acid and, without washing, dry in an oven until it commences to char. Then mount in caustic soda and submit to suitable pressure. The fibre bundles segment into transverse sections, usually between 10 and 20 μ thick, quite flat, exactly transverse and retaining all the fine details of structure of the untreated fibre. The method is a valuable aid to the routine identification of fibres. See Kelaney and Searle, *Proc. Roy. Soc. Lond.*, B, cvi, 1930, p. 357.

1260. Serial Section Mounting. Ribbons or individual sections are most easily handled with the aid of a scalpel, the tip of which is wetted with water.

Paraffin Sections. The albumen and water method of Henneguy is most generally used. For difficult material (moss archegonia, moss capsules, grass leaves, and large sections) use one of the following :—

Land's **Affixative** (*Bot. Gaz.*, lix, 1915, p. 398). Make a 1 per cent. solution of gum arabic in water. Make a potassium bichromate solution immediately before use, adding enough to turn the water pale yellow (about 0·2 per cent.). Smear a few drops of gum arabic solution on the slide and flood with bichromate. After stretching the ribbons, drain off excess water and allow the slide to dry in the light. Exposure to light renders the gum arabic insoluble in water. A mixture of gum arabic solution and

potassium bichromate will not keep. Le Page's glue or Mayer's albumen may replace the gum arabic solution.

SYOMBATHY'S (*Zeit. wiss. Mik.*, xxxiv, 1918, p. 334) gelatin mixture is prepared by dissolving 1 grm. gelatin in 100 c.c. distilled water at 30° C. Add 1 c.c. of a 20 per cent. solution of sodium salicylate, shake well, cool and filter through cheese cloth. Then add 15 c.c. pure glycerin. The mixture should be perfectly clear. Rub 2 drops of the mixture and 2 drops of 2 per cent. formalin on the slide, add the sections and straighten them. The formalin renders the gelatin insoluble. ARTSCHWAGER (*Bot. Gaz.*, lxvii, 1919, p. 373) smears the slide with affixative and floats the ribbons on 2 per cent. formalin solution. After they are stretched, drain off the surplus water and allow to dry in a thermostat in the presence of a small dish of formalin.

HAUPT'S **Modification** (*Stain Tech.*, v, 1930, p. 97). Dissolve 1 grm. of gelatin in 100 c.c. distilled water at 30° C. Add 2 grm. phenol crystals and 15 c.c. pure glycerin. Stir well and filter. The phenol is a better preservative than sodium salicylate. Only the best grade of gelatin must be used and the temperature must not exceed 30° C. Gelatin which dissolves only at a higher temperature is not satisfactory. If one to two days are required for the gelatin to dissolve, decomposition commences, and if the phenol is added too soon the undissolved gelatin turns white and hardens.

BARRATT'S **Collodion-Clove Oil Affixative** (*Ann. Bot.*, xxx, 1916, p. 91); see also BOND (*Trans. Roy. Soc. Edin.*, lvi, 1931, p. 695). A mixture of collodion and clove oil in the proportion of 1 : 3 was used by Barratt. Bond used approximately equal parts. Stretch paraffin ribbons on water on a slide lightly smeared with glycerin to spread water evenly. Transfer the stretched ribbons to a second slide smeared with the affixative. A slight emulsion forms, but it is of no consequence. Allow at least twenty-four hours to dry. The transfer of the ribbons is most easily made with the aid of a pair of broad-tipped, blunt-ended forceps. Grip one end of the ribbon and, with a lifting and pulling motion, raise the ribbon from the water surface; allow the end of the ribbon furthest from the forceps to make first contact with the second slide and then gently lower the remainder of the ribbon into place. The method is especially valuable in permitting the use of eau de Javelle for clearing sections attached to the slide; this reagent commonly destroys other affixing agents.

CRABB (*Science*, lxxx, 1934, p. 530) uses two solutions: (A) a dilute solution of collodion in equal parts of alcohol and ether; (B) a mixture of 1 part amyl acetate with 4 parts of solution A. Spread the sections and dry thoroughly; flood with solution B and leave face up on a level surface until the sections are free from paraffin. Then blot vigorously with filter paper. Next

flood with solution A and stand the slide on end to drain and dry. Harden in 70 per cent. alcohol for several minutes and transfer rapidly through 95 per cent. alcohol into carboxylene or xylol. Leave for several hours and thereafter bring to water.

Collodion sections are usually mounted by the albumen method (§ 216), ether method (§ 217), or by arranging the sections on the slide, gently pressing them and then pouring a little clove oil over them.

1261. Mounting Media. Most commonly used are: 1. **Aqueous mountants,** viz. water, glycerin (pure or diluted), lactic acid, glycerin jelly, Amann's lactophenol, 4 per cent. aqueous formalin, 2 per cent. aqueous acetic acid, Keefe's or Evan's or other fluids that preserve a green colour (but omit any alcohol) and glycerin saturated with zinc iodide. See also Weston, *Science*, lxx, 1929, p. 455 (modified Amann). Aqueous media are satisfactory if the cover is sealed to the slide with a cement. For material of some thickness build up a cell of several well-dried coats of cement and seal the cover to this.

Calcareous material (Chara, etc.) must not be sealed in media containing acids. Mount living marine algæ in media made up with sea-water.

2. **Non-drying oils,** viz. paraffin oil or white mineral oil (after dehydration and clearing), castor oil, linseed oil, olive oil; oil of wintergreen does not require clearing.

3. **Resinous media**, viz. Canada balsam, gum damar, thickened cedar oil, Venetian turpentine, euparal. Styrax, tolu balsam and synthetic resins of high refractive index (see Hanna, *Science*, lxv, 1927, pp. 41 and 575; *ibid.*, lxx, 1929, p. 16; *Journ. Roy. Micr. Soc.*, l, 1930, p. 424; Needham, *Journ. Am. Pharm. Assoc.*, xiii, 1924, p. 424) are most useful for diatoms.

Other mounting media are:—

Potassium silicate (Raybaud, *Rev. Gen. Bot.*, xxxvii, 1925, p. 511); it is hard on most stains: material can be mounted direct from alcohol.

Sodium silicate (Nitzulescu, *C. R. Soc. Biol.*, lxxxix, 1923, p. 1065).

Refer also to Chapter XXI.

1262. Sealing Mounts. Damar balsam, gold size and Venetian turpentine are most commonly used. A mixture of gum mastic and paraffin (Lagerhein, *Bot. Not.*, 1902; see also Thomas, *New Phyt.*, x, 1911, p. 105) is applied by means of a heated thick copper wire. Powder the mastic and heat cautiously in a porcelain dish until melted; add the paraffin in small pieces and stir with a piece of wood until homogeneous and free from lumps. This seal is easily removed with a knife.

Pulverised gum tragacanth (Neumann and Hueber, *Zeit. wiss. Mik.*, xliv, 1927, p. 322) and Duco (Mitchener, *Stain*

Tech., ii, 1927, p. 31) are also recommended. Taylor recommends Hazen's resin-lanolin mixture; melt 8 parts of resin and add 2 parts lanolin.

DIEHL (*Science*, lxix, 1929, p. 276) has an elegant method of sealing non-resinous mounts. Place a drop of the mounting medium in the centre of a large coverslip, and orient the objects in the medium. Cover the mount with a smaller coverslip, and over this place a large drop of fluid Canada balsam. Cover gently with a slide until the smaller coverslip and the mount are surrounded by a ring of balsam and the balsam also covers the exposed under surface of the larger coverslip. Then invert the slide; the object is under the one coverslip and is surrounded by a protected balsam seal.

The balsam tends to run in somewhat, and if water is present in the medium, a cloudy effect is produced. This can be eliminated by carefully painting a thin seal of glycerin jelly around the smaller coverslip and allowing it to dry before applying the balsam. Alternatively seal with immersion oil and ring with balsam.

BARNARD and WELCH (*Journ. Roy. Micr. Soc.*, liv (1), 1934, pp. 29–32). Seal wet preparations.

Using an electrically heated die with the outline of the coverglass which applies melted wax until perfect fusion results. The apparatus has controlled electric heating of wax reservoir, die and feeder tube. The die carries sufficient heat to perfect the seal but not overheat the preparation.

RUYTER (*Bull. d'Histol. Appl.*, xi, 1934, p. 410) has an improvement over Noyer's lake for ringing levulose-gelatin or glycerin preparations. To 100 c.c. liquefied 20 per cent. gelatin in saturated aqueous solution of thyme add 10 c.c. of 5 per cent. $K_2Cr_2O_7$ solution, and mix. Preserve in dark. Allow seal to dry at room temperature in the light.

1263. Mounting Delicate Objects in Resinous Media. Filamentous algæ and other delicate structures usually collapse hopelessly when handled by the ordinary methods. Dehydration and especially clearing and mounting are for them difficult operations. Such material is best stained in aqueous stains and then placed in a 5 per cent. solution of glycerin in water. Allow this to concentrate by evaporation. Then remove the concentrated glycerin completely by several washings of 95 per cent. alcohol and two to three changes of absolute alcohol. Infiltrate with Venetian turpentine or Canada balsam.

1. **Venetian Turpentine Method** (PFEIFFER and WELLHEIM, *Zeit. wiss. Mik.*, viii, 1894, p. 29). Transfer the material quickly from absolute alcohol into a 10 per cent. solution of Venetian turpentine in absolute alcohol, in an open dish in a desiccator over soda lime. Leave here to concentrate. Mount

in the concentrate. The turpentine at 10 per cent. concentration is excessively sensitive to atmospheric moisture, absorbing it and clouding readily. The concentrate is less sensitive. The method eliminates the critical alcohol-xylol transfer; the disadvantages are (1) critical absolute-turpentine transfer, (2) slow hardening of mounts, (3) few suitable stains, (4) a slightly lower refractive index (1·542) than Canada balsam (1·547).

2. **Balsam Infiltration Method** (Taylor). From absolute alcohol, transfer through a series of six to ten alcohol-xylol mixtures (5 : 1, 4 : 2, etc., or 9 : 1, 8 : 2, etc.) five minutes each, and into xylol. Make two to three changes in xylol. Then place in *very dilute* Canada, or better, damar balsam of a concentration a fifth (or less) of that ordinarily used, allow to concentrate, and then mount. The transfer to the balsam solution is critical. Concentration requires two days, rarely more.

CHAPTER XLVII

SOME SPECIAL METHODS MACERATION CLEARING BLEACHING CELL-WALL SUBSTANCES

1264. Maceration Methods. For a full account see KISSER (*Handb. Biol. Arbeitsmethoden* (Abderhalden), Abt., xi, 1931, Teil iv, p. 285).

The living cells of some plants become separated from one another when they are grown in air containing camphor vapour, ether, coal gas and some other substances. Other vapours should be tried. Also plant parts left in solutions of certain nutrient salts become separated into individual cells. Thus root-tips of *Lupinus albus* dissociate after twenty to twenty-four hours in M/100 potassium or magnesium chlorides.

Most methods depend on the ready solution, by various reagents, of the middle lamella, which is usually of a pectic nature. Thus many objects respond to treatment with acid alcohol, followed by ammonia. The principal reagents used are boiling water, freezing, retting, organic acids (malic, citric, oxalic, acetic, tartaric), chromic acid, Schultze's reagent, mineral acids, aqua regia (HNO_3—HCl), strong alkalies, hydrogen peroxide, eau de Javelle.

The epidermis, for hairs and stomata, is stripped mechanically with the aid of a sharp scalpel. Use the leaf or stem in a turgid or but slightly wilted condition. Cut a slit in the epidermis and slip the tip of the scalpel in under the epidermis. In difficult cases scald or boil the leaves in water. Or boil in a 5 per cent. solution of caustic potash until the tissues are translucent and the epidermis separates readily; wash in water.

HEILBORN (*Svensk. Bot. Tidskr.*, xxvii, 1933, p. 161) counts cell numbers or pollen grain numbers in plants by macerating (or powdering) portions and mixing the material with a test substance in which the number of particles per milligram is known.

Macerating Xylem. Here the middle lamella is of lignin. In the cases of herbaceous monocotyledons and ferns first remove the vascular bundles from the ground tissue by hand and cut them into short lengths. Cut perennial woody specimens into longitudinal shavings. It is best to use hot strong (30 per cent. or more) chromic acid; or, chromic acid in conjunction with nitric acid, both at 5 to 10 per cent. strength, either in the cold or heated on a water bath. A more violent method is to boil the material

gently with 50 per cent. nitric acid, adding crystals of potassium chlorate frequently (Schultze's reagent); this should be done in a fume cupboard, well away from microscopes.

Cease the treatment with the macerating agent when the ends of the pieces begin to fray. Wash well in several changes of water and complete the separation by teasing.

Brown (*Bull. Torrey. Bot. Club.*, xlvi, 1919, p. 127) in using Schultze's method, takes equal volumes of acid and water, for safety. Vodrazka (*Zeit. wiss. Mik.*, xliii, 1926, p. 178) treats the material with a specially prepared Schultze's reagent, followed by ammonium hydroxide, or caustic potash or soda. No boiling is necessary; thereby the difficulties of the usual Schultze's reagent are overcome.

Hydrogen peroxide. Use a 30 per cent. solution, and render it weakly alkaline by the addition of a little sodium or lithium carbonate (Kisser, *Cytologia*, ii, 1930, p. 56). It attacks the middle lamella first and the lignin later; finally, after a considerable time, cellulose is attacked. It is useful in isolating parenchymatous tissue.

Eau de Javelle is useful in macerating parenchyma, wood or cork and in isolation of cuticle. It should be fresh, and afterwards the material should be treated with dilute 5 per cent. hydrochloric acid. It attacks lignin first and later the middle lamella; after a considerable time cellulose is attacked.

Harlow (*Bot. Gaz.*, lxxxv, 1928, p. 226) uses chlorination followed by hot sodium sulphite solution which removes the middle lamella and dissolves out the lignin. It is suitable for showing the detail of sieve tubes and vessels.

Aldaba (*Amer. J. Bot.*, xiv, 1927, p. 16) macerates very long fibres of *Bœhmeria* (up to 550 mm.) as follows: Insert the whole stem in a long glass tube, to the lower end of which is attached a flask, and fill the apparatus up with 5 per cent. KOH. Place the base of the flask on a water bath to heat and circulate the liquid. Isolate the fibres in a large shallow dish and float them on to strips of window glass coated with adhesive.

1265. Staining Macerated Wood and Fibres. Boil in safranin or other strong anilin dye, wash and dehydrate rapidly in 95 per cent. and absolute alcohol and clear in xylol, allowing the material to settle before decanting the liquids. Loss of stain is reduced by adding a little xylol to the second and subsequent alcohols. Material cleared without staining can be stained by adding a few drops of clove oil solution of dye to the xylol.

See also Anderson, *Am. J. Bot.*, xiv, 1927, p. 187; Lee, *Bot. Gaz.*, lxii, 1918, p. 318; Tobler-Wolff, *Zeit. wiss. Mik.*, xxxii, 1916, p. 129.

1266. Bleaching may be carried out by the following reagents: hydrogen peroxide (1 part to 4 parts water or 80 per cent. alcohol);

chlorine in 50 per cent. alcohol; eau de Javelle; saturated solution of sulphurous acid in alcohol; dilute potassium permanganate followed by oxalic acid and exposure to light.

1267. Chemical clearing methods are used whenever the presence of the cell contents is unnecessary, in order to make the tissue masses as transparent as possible. Chiefly used are KOH, phenol, chloral hydrate ($CCl_3.CH(OH)_2$) and eau de Javelle (potassium hypochlorite). Prepare eau de Javelle by adding a solution of potassium oxalate to a concentrated aqueous solution of chloride of lime for as long as a precipitate is formed; filter and dilute for use.

Clearing Whole Organs or Thick Sections. SIMPSON (*Stain Tech.*, iv, 1929, p. 131) uses lactic acid, in a concentration of about 75 per cent. Immerse whole flowers or large parts, such as pistils, in open watch-glasses. Mount fairly thick hand-sections of fresh material in acid on a slide. Then place the specimen in a constant temperature oven at about 54° C. until clear. The time required varies; sections of floral parts, young fruits and succulent stems require two to three hours; whole parts or thick wedges of such material take about twelve hours. If permanent slides are desired, allow the lactic acid to thicken slowly in the oven until it is almost hard. Then seal the edges of the preparation with a mixture of gum mastic and paraffin. Clean up slides with the aid of alcohol. See also SESHADI and AIYANGAR (*Indian J. Agric. Sci.*, ii, 1932, p. 51).

BATES (*Amer. Nat.*, lxv, 1931, p. 288) clears leaves by treating them with a saturated solution of chloral hydrate for forty-eight hours, then with potassium chlorate and nitric acid for ten to thirty minutes and finally with potassium chlorate and chloral hydrate for a week. QUICK and PATTY (*Phytopath.* xxii, 1932, p. 925) bleach and clear leaves by processing them, in an airtight container, in an aqueous solution of commercial sodium hypochlorite and lye. MCVEIGH (*Stain. Tech.*, x, 1935, p. 33) uses 5 per cent. sodium hypochlorite solution for clearing leaves. See also: PEACE, *Plant World*, xiii, 1910, p. 93.

STRAIN (*Phytopath.*, xxiv, 1934, p. 82) bleaches leaves and petals quickly by means of alcohol containing nascent chlorine. The material is afterwards cleared, *e.g.* in Amann's medium.

1268. Staining the Vascular Bundles in Whole Organs. BARRATT (*Ann. Bot.*, xxxiv, 1920, p. 201) treats *Equisetum* seedlings with eau de Javelle for twenty-four hours in the cold. Wash in water and stain in ammoniacal fuchsin by Zimmermann's method. Dehydrate, clear with clove oil and mount in balsam. Mounting in glycerin jelly or euparal renders the walls of the parenchyma more visible. Apices and mature shoots may also be successfully treated, but slit these longitudinally into two halves before clearing and mount in a cell with the interior of the stem uppermost.

Caldwell (*Ann. Bot.* xxxix, 1925, p. 212) cuts off the leaf at the middle of the petiole and inserts the cut end in the lower end of a glass tube which is then filled with the stain. The plant is then placed in conditions favourable for transpiration.

Gourley's **Basic Fuchsin Method** (*Stain Tech.*, v, 1930, p. 99). Remove the plants from the soil, wash the roots and immerse them for twenty-four to forty-eight hours in a basic fuchsin solution prepared by dissolving 50 mgm. of the dye in 2 c.c. 95 per cent. alcohol and diluting with 100 c.c. tap-water. After removal from the dye, wash in water and (1) dissect under a binocular microscope after boiling in water or very dilute caustic potash solution. Or, (2) clear by running through the alcohols and then mixtures of alcohol and xylol into pure xylol; this requires ten to twelve hours.

Camp and Liming (*Stain. Tech.*, vii, 1932, p. 91) immerse the cut ends of living plants in a slightly alkaline aqueous solution of basic fuchsin. When the dye has appeared in the parts desired, examine by sectioning, etc. Permanent mounts may be prepared.

Taylor immerses cut ends of vascular plants in eosin solution. If the plant is sufficiently tender, plunge the entire stained shoot in 2 per cent. acetic acid in absolute alcohol to fix the stain and dehydrate the tissues. After a few hours clear in synthetic oil of wintergreen.

1269. Microincineration (see § 678). A thin section of plant tissue carefully heated to destroy the organic matter leaves the ash in its original position and sometimes unaltered. Such an outline of the plant ash constituents is called a *spodogram.* If the heating is continued only long enough to carbonise the tissue, the result is called an *anthracogram.*

During the *carbonisation* of plant sections protected with a cover-glass, the colour of the tissue changes from yellow to red, brown, and finally black. By heating only to the red-brown stage, the material is in the best condition for photomicrography. The distribution of minerals in single cells, as well as in whole tissues, can be presented with a fair degree of accuracy. Silica alone is unaltered by incineration. When alkaline salts are present as well, heating causes the silica to melt and change its form. Remove the alkalies with mineral acids, wash in water and then incinerate. Calcium salts, usually the oxalate, are changed to the carbonate without altering the form of the original crystals.

All kinds of plant material may be used, either fresh or dried. Thick leaves must be sectioned, 20 to 30μ giving the best results. Wood contains relatively few crystals and must be cut thicker. Bark and cortex are commonly crammed with crystals and need to be cut very thinly. The epidermis, usually silicified, is best used alone. Thin sections of coal may be treated similarly. In the best methods, sections on a slide are placed inside a quartz

tube heated externally by a coil of resistance wire; a rheostat in the circuit controls the temperature. The material is heated gently until carbonised and then to a dull red heat for half to one and a half hours. Sections may also be heated on a platinum plate over a Bunsen burner until they are reduced to a white ash. The ash is then transferred without alteration to a slide coated with collodion. The sticky collodion takes up the crystals, which sink into the collodion if the slide is held inverted over vapour of alcohol ether. On drying, the thin film of collodion, with the imbedded crystals, may be removed from the slide with a razor blade and mounted in balsam or an aqueous medium. Ohara transfers the ash from the platinum plate to anilin oil, in which it is immediately ready for study.

See Molisch (*Sitzungsber. Akad. Wiss. Wein* (*Math.-Nat. Kl.*), Abt. I, cxxix, 1920, p. 261); Ohara (*Akad. Wiss. Wein. Math.-Nat. K. Denkschr.*, c, 1926, p. 301); Kisser (Abderhalden *Handb. Biol. Arbeitsmethoden* Abt., xi, Teil, iv, 1931, p. 193); Policard (*Protoplasma*, vii, 1929, p. 464); Policard and Okkels (*Anat. Rec.*, xliv, 1930, p. 349); Scott (*Protoplasma*, xx, 1933, p. 133) (critical review).

1270. Growth of Cell-wall is studied by precipitating a stain in the wall. Nell (*Wurzburger Habilitationsschrift Abhandl. Senckenburg nat. Ges.*, xv, 1887, p. 101) used a precipitate of Berlin blue (potassium ferrocyanide and ferric chloride) or of Turnbull's blue (potassium ferricyanide and ferrous lactate) in the walls of marine algæ such as *Caulerpa*. Immerse the alga, for a few seconds, in a mixture of 1 part sea-water, 2 parts fresh water and sufficient potassium ferrocyanide to give the solution the density of sea-water. Rinse rapidly in sea-water and immerse half to two seconds in a mixture of 2 parts sea-water, 1 part fresh water and a few drops of ferric chloride. Repetition intensifies the colour, which, however, is gradually destroyed afterwards.

Zacharias (*Flora*, 1891, p. 467) and Klebs (*Untersuch. bot. Inst. Tübingen*, ii, p. 489) have used Congo red.

For the daily growth rings in cotton hairs, Balls (*Proc. Roy. Soc. Lond.*, B, xc, 1919, p. 542) wells them to five to ten times their normal size by treatment with NaOH and carbon bisulphide.

1271. Finer Structure of Cell-walls. See especially Correns (*Pringsheim's Jahrb. wiss. Bot.*, xxiii, p. 254). The fine sculpturing may be observed in media of low refractive index, *e.g.* methyl alcohol ($\mu = 1{\cdot}321$). Differentiations due to unequal water-content require drying at 100° C., as absolute alcohol, and other dehydrants do not give positive results. Afterwards impregnate the wall with Berlin blue or silver. For the latter, place well-dried objects in 2 to 5 per cent. aqueous silver nitrate, dry superficially but do not wash, and then place in 0·75 per cent. aqueous NaCl. Reduce the AgCl thus precipitated by exposure

for several hours to full sunlight. See ALVARADO (*Bol. R. Soc. Española Hist. Nat.*, xix, 1919, p. 66 and *Trab. Mus. Nacion Cien. Nat. Ser. Bot.*, No. 13, 1918, p. 9) for an application of the tannin-silver method of Achúcarro-Río Hortega (§ 1094).

1272. Micellæ. For methods of study using polarised light see, for instance, BALLS, *Proc. Roy. Soc. Lond.*, B xcv, p. 72; FREY, *Jahrb. Wiss. Bot.*, lxv, 1926, p. 195; *Naturwiss.*, xv, 1927, p. 760; *Ber. Deut. bot. Ges.*, xliv, 1926, p. 564; *Protoplasma*, xxxiv, 1928, p. 139.

Cell-wall Substances: Some Microchemical Tests and Stains. For a general account see VAN WISSELINGH, *Die Zellmembranen*, in LINSBAUER'S *Handb. Pflanzenanat.*, iii, 2, 1925; also, BROWN, *Bull. Torrey Bot. Club*, xlvi, 1919, p. 127; HAAS and HILL, "Chemistry of Plant Products," Longmans, 1928; SCHORGER, "Chemistry of Cellulose and Wood," McGraw-Hill, 1926, and others.

1273. Cellulose swells and dissolves rapidly in concentrated sulphuric acid.

A blue colour is obtained by treatment with iodine and strong sulphuric acid (SCHLEIDEN, *Pogg. Ann.*, xliii, 1838, p. 391). Soak in a solution of 0·3 grm. iodine and 1·3 grm. KI in 100 c.c. water, followed by a few drops of 60 to 70 per cent. sulphuric acid. Lignin turns somewhat more yellow than before.

It stains blue with chlor-zinc-iodide (see VAN WISSELINGH, *Jahrb. Wiss. Bot.*, xxxi, 1897, p. 624). This reagent is also known as the Herzberg iodine stain. Prepare as follows: Dissolve 50 grm. of dry zinc chloride (fused sticks) in 25 c.c. distilled water, adjusting the specific gravity to 1·8, and pour 40 c.c. into a tall cylinder. Dissolve 5·25 grm. KI and 0·25 grm. iodine in 12·5 c.c. water and add to the zinc chloride solution. Mix well and place in the dark. After twenty-four hours pipette off the clear portion into a black bottle, and add a leaf of crystalline iodine. The solution deteriorates rather rapidly so where colour differentiations are important do not use longer than two weeks.

A two-solution method due to NOVOPOKROWSKY (*Beih. bot. zbl.*, xxviii, 1912, p. 90) gives uniformly good results. Stain for a few seconds in a 1 : 1 : 100 solution of iodine and potassium iodide in water. Then transfer to a solution of 2 parts zinc chloride in 1 part of water, where the section is kept moving until a light blue colour is produced. See also ARTSCHWAGER, *Bot. Gaz.*, lxxi, 1921, p. 400; and WIGHTMAN, *Paper*, xxxi, 1923, p. 14.

MANGIN (*Compt. rend*, cxiii, 1891, p. 1069; *Bull. Soc. Bot. France*, xxxv, 1888, p. 421) found iodine and phosphoric acid colour cellulose blue-violet, while iodine and calcium chloride give a rose or violet colour. Iodine with many other salts ($AlCl_3$, $CaCl_2$, $MnCl_2$, $SnCl_2$, $Ca(NO_3)_2$, and $Zn(NO_3)_2$) stains cellulose, but $ZnCl_2$ is most sensitive.

See also MANGIN (*Compt. rend.* cxi, 1890, p. 120) for other stains. Among them Congo red gives a characteristic reaction for cellulose; the red-stained cell-wall is turned blue by steeping in dilute HCl (DORNER, *Zentralbl. Bakt. Parasitenk. Infektionskrankh.* 2, lvi, 1922, p. 14).

Schweizer's reagent (cuprous ammonia) is also a specific reagent (see GILSON, *La Cellule*, ix, 1893, p. 404). It dissolves the cellulose out of the wall. Subsequent dilution with ammonia causes the cellulose to separate out in the cell lumina. To prepare the reagent add 13 to 16 per cent. ammonia to copper turnings and allow to stand in an open bottle. Or, precipitate cupric oxyhydrate from a solution of cupric sulphate by addition of dilute NaOH, wash the precipitate and dissolve it in strong ammonia.

1274. Oxycellulose. Most tests are based on the property of reduction and therefore do not differentiate oxy- from hydrocellulose. Knagg's test (*J. Soc. Dyers and Colourists*, xxiv, 1908, p. 112) is most certain. Treat sections with HCl, wash in water, dye deeply with benzopurpurin (or Congo red), acidify with HCl (changing the red colour to blue) and wash in tap-water. Oxycellulose stains blue, cellulose and hydrocellulose deep crimson.

SCHWALBE and BECKER (*Deut. Chem. Ges. Ber., Jahrg.*, liv, 1921, p. 545) immerse sections in methyl orange and afterwards in concentrated brine. Oxycellulose becomes a deep red, cellulose and hydrocellulose remain yellow.

See also WOOD, *Ann, Bot.* xxxviii, 1924, p. 273; xl, 1926, p. 547; MEHTA, *Biochem. Journ.*, xix, 1925, p. 979; KNECHT and THOMPSON, *J. Soc. Dyers and Colourists*, xxxvii, 1921, p. 271.

1275. Lignin stains a golden yellow colour with anilin sulphate (or chloride) (RUNGE, *Ann. Physik. Chem.*, xxxi, 1834, p. 65). The colour is more intense if followed by sulphuric acid; or use an acidified solution. Phloroglucin followed by 50 per cent. HCl gives a red-purple coloration (VON HÖHNEL, *Sitzungsber. Ak. Wiss. Wien.*, lxxvi, 1877, pp. 528, 663). STECÓWNA (*Acta Soc. Bot. Poloniæ*, iii, 1925, p. 138) uses methyl red for lignified walls. Add enough alkali to a 0·001 per cent. solution to render it yellow. Lignified walls are stained red because of their acidity (pH 5) and the colour is permanent.

Ungar's diazonium reaction (*Diss. Zürich*, 1914, p. 23). Place sections in dilute sodium carbonate solution and add a little diazonium chloride solution. In a short time a brick-red colour results.

Iodine followed by strong sulphuric acid gives a brown colour. Lignin swells, carbonises and slowly dissolves in concentrated sulphuric acid. Eau de Javelle and other macerating fluids, change it to cellulose, when von Höhnel's reaction is no longer given. The walls then react like cellulose, swelling and dissolving rapidly in concentrated sulphuric acid.

A dull brown colour is produced by KOH, and a deep brown colour by chlor-zinc-iodide.

Most lignin reactions appear to be due to bodies of an aldehyde nature (probably coniferyl aldehyde). For the lignin complex itself, Cross and Bevan's chlorine—sodium sulphite reaction (*J. Chem. Soc.*, lv, 1889, p. 199) seems most reliable (see SCHORGER, *J. Ind. Eng. Chem.*, xv, 1923, p. 812). Exposure to moist chlorine gas or bromine or treatment with chlorine water produces a yellow colour, changed to red on the addition of sodium sulphite. In the Mäule reaction (*Fünfstuk's Beitr. wiss. Bot.*, vi, 1900, p. 166) material is first treated with oxidising agents. For example, treat with 1 per cent. potassium permanganate five minutes, then with HCl and wash with water and add ammonia. A bright red coloration results; the wood of Gymnosperms, however, usually gives a brownish-grey.

For a summary of numerous other reactions see VAN WISSELINGH and the references there quoted. See also CROCKER, *J. Ind. Eng. Chem.*, xiii, 1921, p. 625; ABRAMS, *J. Ind. Eng. Chem.*, xiii, 1921, p. 786.

1275 ***bis*****. Pectic Substances** occur principally as the calcium salt of pectin in the cell-walls of higher plants, especially in the middle lamella. They are soluble in macerating liquids and (especially in fruits) in boiling water and very dilute acids. They are soluble also in ammonium oxalate and in HCl followed by KOH; the latter method requires careful management.

Neutral violet stains pectin a brown-red colour.

Ruthenium red (see MANGIN, *Bull. Soc. Bot. France*, xli, 1894, p. 40) does not stain pure cellulose and possesses more affinity for pectic substances than any other stain used in botanical technique. It is best used in neutral or slightly ammoniacal solution. Ruthenium red, however, also stains gums, mucilages (MANGIN; TUNMAN, *Pflanzenmikrochemie*, 1913), fatty acids (TUPPER CARY and PRIESTLEY, *Proc. Roy. Soc. Lond.*, cxv, 1923, p. 109), gelose (TUNMAN), etc.

Treatment with Schweizer's reagent by the method of FRÉMY (*Compt. rend.*, lxxxiii, 1876, p. 1136) removes the cellulose and leaves the pectic substance of the cell-wall as an insoluble framework, which can be stained with ruthenium red (CARRÉ and HORNE, *Ann. Bot.*, xli, 1927, p. 193).

See also HOWE, *Bot. Gaz.*, lxxii, 1921, p. 313.

Affinity of Cellulose, Lignin and Pectic Substances for Stains. MANGIN (*Compt. rend.*, cix, 1889, p. 579; cx, 1890, pp. 295 and 644) finds that pectic substances, lignified and suberised walls stain with methylen blue, Bismark brown and fuchsin, while pure cellulose does not. If such stained sections are treated with alcohol, glycerin or dilute acids the pectic substances are decolourised rapidly, whereas the lignin and suberin retain their

coloration. Crocein and nigrosin stain lignified and suberised walls, but not the pectic substances. Crocein, naphthol black, orseille red, Congo red and azo-blue stain pure cellulose but not pectic substances.

See also MEHTA, *Biochem. Journ.*, xix, 1925, p. 979 and section on Staining.

1276. Mucilage and mucilaginous walls swell in water, are insoluble in alcohol and ether and are readily stained with methylen blue and gentian violet. Slimes may sometimes be differentiated by colour reactions, though they are often mixtures. See, especially, MANGIN, *Bull. Soc. Bot. France*, xli, 1894, p. 40.

1277. Cellulose derivatives hardly react to iodine. They stain with alkaline solution of Congo red.

1278. Pectose derivatives stain brown with lead acetate and with alum. They are stainable with neutral solutions of hæmatoxylin, methylen blue, neutral red and ruthenium red.

1279. Callose derivatives, which are readily soluble in dilute NaOH or KOH, stain with an acid solution of anilin blue.

1280. Gums swell and dissolve in water, are soluble in alcohol and are stained red with an ammoniacal solution of ruthenium red. Many stain well with corallin soda, anilin blue and anilin violet.

1281. Suberin and cutin are unaffected by eau de Javelle and are comparatively insoluble in H_2SO_4 and in chromic acid. Suberin becomes invisible in the latter reagent owing to its dark colour, but requires several days for solution. Both give a yellow-brown colour with iodine followed by sulphuric acid. Phloroglucin, followed by HCl, produces a deep pink colour. Concentrated aqueous KOH gives a bright reddish-yellow colour with suberin, and a bright yellow colour with cutin; the colours are somewhat intensified on warming (VON HÖHNEL, *Sitzungsber. Ak. Wiss. Wien.*, lxxvi, 1877, p. 507). They turn yellow-brown with chlor-zinc-iodide, especially in sections treated with eau de Javelle and washed with 1 per cent. HCl (VON HÖHNEL, l. c.). They are stained red by alcoholic solutions of alkanin, Sudan III, and Scharlach R., and are also stained by chlorophyll (see ZIMMERMANN, *Zeit. wiss. Mik. mik. Tech.*, ix, 1892, p. 58; SOAR, *New Phyt.*, xxi, 1922, p. 269). Neutral violet (1: 10,000 aqueous solution slightly acidified) is said to stain suberin violet, but not cutin.

They are usually stained similarly to lignin by ordinary histological methods. Stains which, in addition to colouring lignified walls and cork sharply differentiate cutinised cellulose, are in order of merit: Magdala red (genuine), methyl green, solid green, malachite green, gentian violet and fuchsin. Less useful are: safranin, methyl blue, neutral red, erythrosin and eosin. The stains are best used in dilute aqueous solution. To correct over-

staining or the staining of other tissues wash in alcohol, used pure or slightly acidulated with HCl. See Kisser, *Zeit. wiss. Mik.*, xlv, 1928, p. 163; Tison, *C.R. Assoc. Franc. Av. Sci.*, xxviii, Pt. 2, 1899, p. 454; Priestley, *New Phyt.*, xx, 1921, p. 17.

Cutin is very resistant to acid hydrolysing agents, but is readily oxidised by either acid or alkaline oxydising agents. The best stain for it is 1 per cent. alcoholic Sudan III.; heat sections on the slide until the alcohol boils. For delicate cuticles use Nile blue sulphate or dimethylaminoazobenzene. See Lee and Priestley, *Ann. Bot.* xxxviii, 1924, p. 525.

1282. Callose (see Mangin, *Compt. rend.*, cx, 1890, p. 644) occurs principally in sieve plates of the phlœm, in pollen grains and tubes and in fungal hyphæ. It is insoluble in water, alcohol, cold alkali carbonates, ammonia and Schweizer's reagent, but very soluble in 1 per cent. NaOH or KOH, concentrated H_2SO_4 and concentrated calcium chloride and tin chloride solutions. Iodine colours it yellow, chlor-zinc-iodide brick red or red brown. It is strongly stained by a dilute solution of corallin in 4 per cent. aqueous sodium carbonate, by dilute aqueous anilin blue followed by dilute HCl, and by a number of other anilin dyes, especially after alkali treatment or oxidation.

1283. Chitin is a constituent of fungal cell-walls. Use Van Wisselingh's (*Jahrb. Wiss. Bot.*, xxxi, 1898, p. 657) chitosan reaction. Seal material in glass tubes with glycerin and heat to 300° C. in an oil bath. Then treat in concentrated or 50 per cent. KOH at 160° to 180° C. for twenty minutes and wash on slide with 95 per cent. or absolute alcohol. Bring to distilled water, treat with aqueous iodine solution and then with 1 per cent. H_2SO_4. Chitinous walls then stain violet red, the colour disappearing in 70 per cent. acid. If cellulose is present a blue colour then develops.

A less intense blue-violet colour is obtained with iodine—potassium iodide solution and zinc chloride or chlor-zinc iodide. See § 1183; see also Hopkins, *Trans. Wis. Acad. Sci.*, xxiv, 1929, p. 187.

1284. Chloramine Test for Nitrogen in Cell-wall (Wood, *Ann. Bot.*, xl, 1926, p. 1; xli, 1927, p. 281). Submit sections to chlorine gas for a prolonged period and treat with sodium phosphate and potassium iodide solutions. The presence of protein is indicated by the liberation of iodine. In lignified tissues the colour passes from yellow to brown and finally to pink.

1285. Latex and Latex Vessels. Material containing latex must be collected in a manner to minimise loss of the latex before fixation is effected. Where there are long latex tubes, ligature the stem near the places where it is to be cut and preserve in 50 per cent. alcohol without removal of the ligature until hardening is complete. Longitudinal sections are usually best. Stain in a dilute aqueous iodine solution, followed by aqueous eosin or

erythrosin. The tubes stain rose-pink, the starch-grains purple. Mount in 2 per cent. aqueous acetic acid, or dehydrate, clear and mount in balsam. Latex vessels and cells are well brought out by a heavy stain of safranin.

Zijp (*Arch. Rubbercult. Nederland-Indie*, iv, p. 65) uses Sudan III. for staining latex vessels.

Popovici (*C. R. Acad. Sci.*, Paris, clxxxiii, 1926, p. 143) stains with neutral red, cresyl blue and indophenol blue.

1285 *bis*. Sieve-tubes. The coagulable protein materials in sieve-tubes are shown up by fixation in alcohol, but their distribution is generally not properly maintained. A. Fischer (*Ber. Deut. bot. Ges.*, 1885, p. 230) plunges whole plants, or long portions of plants such as branch-tips, into boiling water for two to five minutes and then cuts them up for preservation. This coagulates the contents rapidly and the "Schlauchköpfe," artifacts due to cutting, are not obtained.

Stain half an hour or longer in dilute aqueous anilin blue solution, rinse in water and dehydrate to 70 per cent. alcohol; counter-stain with 1 per cent. eosin or erythrosin in 70 per cent. alcohol, dehydrate, clear and mount. The anilin blue is intensified by treatment with mild alkali (ammonium hydroxide or carbonate). It needs no differentiation. If desired, sections can be placed in a stronger solution and afterwards differentiated in glycerin. Callose blue, rest pink. (Russow, *Sitzungsber. nat. Ges. Univ. Dorpat.*, vi, p. 63).

A saturated aqueous solution of eosin followed by a 1 per cent. solution of methylen blue in 90 per cent. alcohol also gives a good stain. Eosin fifteen to twenty minutes, wash in dilute alcohol, stain in methylen blue one to two minutes, dehydrate rapidly in absolute and clear with clove oil. This is also good for latex, medullary rays, the development of sieve tubes and for conducing parenchyma.

Harrar (*Bot. Gaz.*, lxxxvi, 1928, p. 111) recommends Bismark brown and Heidenhain's hæmatoxylin for the phlœm tissues of woody plants.

1286. Surface features of plants may frequently be studied by reflected light. *Neuwirth's Casting Method* (*Zeits. Zuckerind. Czechoslovak. Republ.*, liv, 1930, p. 341). Place plants in an ether-alcohol and acetic acid solution of collodion with addition of castor oil or paint on a film with a brush. The mixture is coloured by a highly concentrated acetic-acid-fuchsin solution. Strip off film as soon as it dries at the edge. The surface details of roots, stems and leaves and numbers of stomata per square millimetre of leaf surface, the latter by means of miniature quadrats and transects, may be studied. Long and Clements (*Amer. Journ. Bot.*, xxi, 1934, p. 7) also use a solution of cellulose acetate. Artsikhovskaia (*Journ. Bot. U.S.S.R.*, xvii, 1932,

p. 154) uses a 10 per cent. gelatin solution. See also OHGA, *Bot. Mag. Tokyo*, xl, 1926, p. 550.

1286 *bis*. Preservation of Natural, especially Green, Colour. The simplest fluid consists of concentrated formalin saturated with copper acetate, diluted to 4 per cent. for use. This is especially useful for Chlorophyceæ and Myxophyceæ. The green colour is more bluish than the original and there is usually some shrinkage.

SEMICHON (*Rev. Path. Veg. et Ent. Agric.*, xiv, 1927, p. 228) suggests water 8 c.c., 40 per cent. formalin 1 c.c., 4 per cent. aqueous solution of copper acetate 1 c.c. EVANS (*Journ. Quekett Micr. Club.*, xiv, 1921, p. 225) suggests fixation in a mixture of 10 c.c. of 5 per cent. neutral formalin and 1 c.c. of 10 per cent. zinc acetate in thymol water, diluted if shrinkage occurs. After a time the material is washed and preserved in glycerin by concentration. KEEFE (*Science*, lxiv, 1926, p. 331) gives :

Fifty per cent. alcohol	90 c.c.
Commercial formalin	5 ,,
Glycerin	2·5 ,,
Glacial acetic acid	2·5 ,,
Copper chloride	10 grm.
Uranium nitrate	1·5 ,,

This acts as a fixing and preserving fluid, causes little or no shrinkage and gives a natural green colour. For Myxophyceæ, substitute 10 grm. copper acetate for the copper chloride and uranium nitrate. For yellowish-green plants reduce the copper chloride to half. Delicate forms are fixed in forty-eight hours, others in three to ten days. In making microscopic mounts transfer to a mixture from which the alcohol has been omitted.

JIROUCH (*Stain Tech.*, iv, 1929, p. 17) fixes plant tissue at least forty-eight hours in Keefe's fluid. Wash well in water. Steep in 20 per cent. gum arabic for one to two days and cut after freezing when the mass has just started to melt. Stain lightly in Delafield's hæmatoxylin (5 per cent. for about two minutes). Blue in distilled water containing a little sodium carbonate. Dehydrate in glycerin and mount in glycerin jelly.

NEUMAYER (*Ber. Deut. bot. Ges.*, xl, 1922, p. 41) fixes fronds of *Angiopteris* in hot concentrated potassium bichromate solution. The green colour of the chloroplasts is retained after dehydrating, imbedding and mounting in the usual way.

See also MALTBY, *Mus. Journ.*, xxv, 1926, p. 329 ; SEIF-EL-NASR, *Min. Agr. Egypt., Tech. and Sci. Serv. Bull.*, cxxiv, 1932, p. 1 ; NIEUWLAND and SLAVIN, *Proc. Indiana Acad. Sci.*, xxxviii, 1929, p. 103 (preservation of *Monotropa*, etc., without discoloration).

CHAPTER XLVIII

GENERAL STAINING

1287. The stains used are mostly the standard solutions of single dyes. Aqueous solutions, *e.g.* of eosin, carmine, gentian or crystal violet, methylen blue, anilin blue, picric acid, are commonly 1 or 2 per cent. solutions in distilled water. Solutions of crystal or gentian violet in anilin water (saturated aqueous solution of anilin oil, prepared by shaking and filtering) stain more densely but do not keep so well. The hæmatoxylins most used are Heidenhain, Delafield, Erhlich and Kleinenberg. Alcoholic solutions are generally 1 per cent. and are made up in 70 per cent. alcohol, *e.g.* fuchsin, light green, methyl or iodine green, Bismark brown, crystal or gentian violet, alcoholic eosin or erythrosin, auramine, phloxine (Magdala red). Safranin O is usually used as a 1 per cent. solution in 50 per cent. alcohol, prepared with distilled or anilin water (Zwaardemaker, *Zeit. wiss. Mik.*, iv, p. 212). Taylor adds 0·25 per cent. auramine to solutions of safranin and light green if the colour tone is too cold.

Freehand sections can usually be well stained using much shorter periods in the stains than are indicated for microtome sections. The length of time in the stains depends upon the material as well as the strength of the stain solution.

Wherever possible, especially when using combination stains, the course of the operations should be closely watched under the microscope. Differentiation and counterstaining may then be stopped at the critical stage. This is especially important when a second stain tends to reduce the primary one. In such cases, overstain with the first stain and cease the differentiation of it before the correct point has been reached.

When the differentiation with an aqueous or alcoholic counterstain is difficult, owing to very great loss of stain, dehydrate the sections to 95 per cent. alcohol and differentiate the primary stain. Then clear with a solution of the counterstain dissolved in clove oil. Crystal (or gentian) violet, erythrosin, light green and orange G may be employed thus. This method of staining, however, appears merely to paint a coat of the dye on the unstained, non-lignified tissues. The staining is usually brilliant but the differentiation dubious.

See also Earl, *Science*, lxxii, 1930, p. 562 (butyl alcohol procedure).

For the effect of *p*H on differential staining, see NAYLOR, *Amer. Journ. Bot.*, xiii, 1926, p. 265.

Material mounted in glycerin jelly may be stained by adding small amounts of stain to the jelly.

Pre-staining before sectioning is little used; see OTIS, *Science*, lxvi, 1927, p. 196 (alcohol-xylol-safranin method).

McLEAN (*New Phyt.*, xxxiii, 1934, p. 316) stains material with the free acids of erythrosin and eosin dissolved in xylol. Dissolve 1 grm. of stain in 100 c.c. water and add 10 per cent. HCl (s.g. 1·16), 3·5 c.c. for the erythrosin and 2·5 c.c. for the eosin. Allow the precipitate to settle and after twenty-four hours pipette off the clear liquid. Add 200 c.c. xylol and shake very gently or stir with a glass rod; avoid an emulsion. Separate off the (nearly colourless) xylol extract. Stain with it after clearing in xylol. The free base of methylen blue (Nile blue is better) can be used similarly. Precipitate it by adding 2·5 c.c. 10 per cent. NaOH for each gram of the dye. The two xylol stains can be used successively, but not mixed.

See STEVENS and HAWKINS, *Journ. Agr. Res.*, vi, 1916, p. 362.

Single stains are usually preferable for apical cells, young antheridia, archegonia and other relatively undifferentiated structures, if the preparations are for anatomical study. In such cases, stains which deeply colour the cell-walls without obscuring them by also colouring starch, plastids and other cell contents, should be used. Further, it is better *not* to counterstain the cytoplasm in these cases, as the dense cytoplasm often takes a heavy stain and obscures the walls.

Combination stains have a wide application for differentiation between elements that are lignified or suberised and those that are not. It is usually best to combine a basic with an acid stain, the former for lignified and suberised, the latter for cellulose walls. A great number of stain combinations have been employed; some of the more widely used and successful ones are described below.

1288. Staining of Ribbons of Sections without Removal of Paraffin. Stretch ribbons as usual and float them upon the surface of the stains and then distilled water. Float them into place upon the surface of an albumenised slide, dry, clear and mount. Sections of the same tissues may be stained by different methods and mounted side by side for comparison. The removal of the paraffin is necessary only in case a very narrow diaphragm opening is used.

Forceps with broad, flat, blunt ends about 2 to 4 mm. wide are useful for picking up the ribbons. The end of the ribbon may be gripped with such an instrument and the whole ribbon easily lifted by a gentle pulling motion.

See McFARLAND, *Science*, lvi, 1922, p. 43; DE FRAINE, *New Phyt.*, xii, 1913, p. 123.

ANATOMICAL STAINS

1289. Basic fuchsin is one of the best for lignified and suberised tissues. Prepare by adding a 5 per cent. alcoholic solution to strong ammonia (0·880) so long as the solution remains colourless or has merely a pale straw colour. To use (1) immerse freehand sections in the stain for some time and then wash in 95 per cent. or absolute alcohol until the correct depth of colour has been produced, then clear in clove oil (ZIMMERMANN). (2) Dip sections into the stain for a few seconds and then expose to allow the ammonia to evaporate; when the correct colour has been reached dehydrate rapidly and clear (BOND, *Trans. Roy. Soc. Edin.*, lvi., 1931, p. 695).

1290. HEIDENHAIN'S **Iron Hæmatoxylin,** followed by safranin as a counterstain, is a good combination for general anatomical purposes, and especially for wood sections. Congo red (saturated aqueous solution) is superior to safranin as a counterstain for bark.

1291. Safranin and DELAFIELD'S **Hæmatoxylin.** Stain in safranin three to twenty-four hours (or less) and differentiate in 50 per cent. alcohol, acidulated if the stain comes out too slowly, until the parenchyma is pink, while the lignified tissues are red. Wash in distilled water. Stain in Delafield's hæmatoxylin until the stain is sufficiently dark. Wash in several changes of slightly alkaline water (tap water or distilled water with a trace of ammonia). A poor contrast results from insufficient washing. Collenchyma and plastids purple-blue; lignified tissues, cuticle, cork or nuclei red.

1292. HEIDENHAIN'S **Iron Hæmatoxylin and Eosin** (or **Erythrosin**). Stain in the hæmatoxylin and dehydrate to 70 per cent. alcohol. Counterstain with eosin or erythrosin. Cellulose, etc., pink; lignified tissues and nuclei black. Depending upon the degree to which the differentiation is carried, the hæmatoxylin may stain the whole wall of lignified tissue or be restricted to the middle lamella.

1293. DELAFIELD'S **Hæmatoxylin and Erythrosin.** Stain in hæmatoxylin and afterwards stain thirty seconds to one minute in erythrosin. Orange G is also a good stain to follow Delafield's hæmatoxylin.

1294. Safranin and Light Green. Stain in safranin and differentiate. Stain in a solution of light green in 90 per cent. alcohol for ten to thirty seconds. The light green stains vigorously and also reduces the safranin. Pass rapidly through 95 per cent. and absolute alcohols and clear in xylol. The cellulose tissues stain green. The light green can also be dissolved in clove oil. Stain not more than thirty seconds, rinse with clove oil and wash with xylol.

Methyl green or iodine green may be used in place of the light green. It is less easy to obtain a clear green colour with them and they are less permanent. Parenchyma containing tannin frequently holds the green stain well. The combination is unsuitable for tissues weakly lignified.

A violet staining of the cellulose tissues is obtainable using an aqueous, alcoholic or clove oil solution of crystal or gentian violet in place of the light green, and an orange stain by the use of orange G, in either alcohol or clove oil solution.

1295. Safranin and Anilin Blue. Stain in safranin and partially differentiate. Stain in alcoholic anilin blue one to ten minutes and differentiate briefly in 95 per cent. alcohol. Then fix and intensify the blue stain with slightly acidulated (HCl) alcohol. Wash in neutral 95 per cent. alcohol, followed by absolute, and clear in xylol. The cellulose tissues stain a brilliant blue. The combination is difficult and the contrast unsuitable for microphotography.

The use of auramine in place of the safranin gives an easier combination and a less severe contrast.

1296. Crystal (or Gentian) Violet and Bismark Brown. Stain in aqueous violet solution ten to twenty minutes, and without washing stain in Bismark brown (in 80 per cent. alcohol) for about five seconds. Dehydrate rapidly in absolute alcohol and differentiate in clove oil. Wash in xylol. A brilliant stain: lignified tissues, cuticle and cork violet, cellulose tissues brown.

Orange G, in alcoholic or clove oil solution, may be used in place of the Bismark brown to give an orange stain in the cellulose tissues.

WARINGTON (*Ann. Bot.*, xl, 1926, p. 27) uses gentian violet (saturated solution in 40 per cent. alcohol) for ten minutes, followed by Vesuvian brown (saturated solution in 50 per cent. alcohol) for a few seconds.

1297. Iodine Green and Acid Fuchsin. Stain in aqueous iodine green and differentiate briefly with 95 per cent. alcohol. Counterstain with acid fuchsin for two to three minutes. Differentiate in absolute alcohol. Lignified tissues, cuticle and cork green, cellulose tissues pink. The difficulty lies in losing too much green, but the combination is useful in giving green xylem where the latter is too dense to photograph when red. The stock solution of acid fuchsin is often best diluted with 1 to 5 parts of 70 per cent. alcohol. Methyl green (aqueous solution) can be used in place of iodine green.

1298. Bismark Brown and Light Green. Stain lightly with Bismark brown and differentiate in 50 per cent. alcohol. Counterstain with alcoholic light green for one to five minutes and differentiate in 70 per cent. alcohol. Lignified tissues brown, cellulose green. Differentiation not always very sharp.

1299. Picric Acid and Methylen Blue. Stain twenty-four hours or more in saturated aqueous picric acid, rinse quickly in water and stain briefly in aqueous methylen blue. Rinse and dehydrate rapidly. Lignified tissues, etc., lemon yellow, cellulose tissues blue.

1300. Picric Acid and Fuchsin. Pujiula (*Bol. Soc. Iberica Cienc. Nat.*, i, 1928) gives a schedule for picric-fuchsin used in mixture. The combination is useful for Monocotyledon stems in which the stelar and cortical tissues are hard to differentiate.

1301. Crystal violet and erythrosin (Jackson, *Stain Tech.*, i, 1926, p. 33) is useful for material in which the xylem elements are small or weakly lignified. Stain in 1 per cent. crystal violet, in distilled water, for fifteen minutes, rinse quickly in water, dehydrate rapidly in 95 per cent. and absolute alcohol. Stain one to five minutes in a saturated solution of erythrosin in clove oil, treat with equal parts absolute alcohol and xylol one to two minutes and clear in xylol. Erythrosin tends to replace crystal violet in lignified tissues, hence its action needs exact control.

1302. Phloxine (or Magdala Red) and Anilin Blue. Stain in phloxine (1 per cent. in 90 per cent. alcohol) three to twenty-four hours, rinse in 95 per cent. alcohol and stain two to ten minutes in anilin blue (1 per cent. in 90 per cent. alcohol), made up freshly. Rinse in 95 per cent. alcohol and treat with 95 per cent. alcohol slightly acidulated with HCl. The blue is intensified and the red extracted. Wash in 95 per cent. alcohol rendered slightly alkaline with sodium carbonate if the red has become too dull. Wash in absolute alcohol and clear in xylol. See Chamberlain, *Stain. Tech.*, ii, 1927, p. 91.

1303. Safranin and Picro-anilin Blue (Smith, *Science*, lix, 1924, p. 557). Stain in safranin two hours, and destain to a light pink in 50 per cent. alcohol. Stain two hours in picro-anilin blue (saturated solutions, in 95 per cent. alcohol, of picric acid and anilin blue mixed in ratio of 78 per cent. picric acid to 22 per cent. anilin blue.) Wash ten seconds in absolute alcohol and clear in clove oil.

1304. Malachite Green (or Methylen Blue) and Congo Red. Used by Gregoire (see Chamberlain, p. 94). Stain in 3 per cent. aqueous malachite green for six hours or more, wash in water and stain in 1 per cent. aqueous Congo red for fifteen minutes. Wash in water and differentiate in 80 per cent. alcohol until the green stain appears through the red. Dehydrate rapidly in absolute alcohol and clear in xylol.

1305. Cyanin and Erythrosin. Best used made up in 50 per cent. alcohol. Stain in cyanin five to ten minutes or longer, rinse quickly in 50 per cent. alcohol and stain thirty seconds to one minute in erythrosin. Rinse quickly in 50 per cent. alcohol, then

95 per cent. alcohol and absolute and clear in xylol. Lignified tissues blue, cellulose tissues red.

Aqueous solutions may also be used, but the stains then wash out even more rapidly.

1306. MALLORY's **Triple Stain** is recommended by PUJIULA (*Brotéria, Sér. Bot.*, xxv, 1931, p. 51) for *Pinus* sections. He obtained the best results by fixation in 90 to 95 per cent. alcohol and the substitution of Ziehl's basic fuchsin for the acid fuchsin.

1307. Polychrome Methylen Blue is a quick and easily controlled stain, differentiating well. Stain and then mordant for a few minutes in 10 per cent. aqueous ammonium molybdate before dehydrating (BARRATT, *Ann. Bot.*, xxx, 1916, p. 92).

1308. Light Green and Sudan III (BUGNON, *C. R. Acad. Sci.*, Paris, clxviii, 1919, p. 62). Stain in saturated aqueous or alcoholic light green, acidulated with hydrochloric or acetic acid. Wash in water to remove stain from all but the lignified tissues and then stain in an alcoholic solution of Sudan III. Bugnon suggests some triple combinations, involving ammoniacal gentian violet or other stains in addition.

1309. Staining Cell-walls in Meristematic Tissues. FOSTER (*Stain Tech.*, ix, 1934, p. 91) describes the use of tannic acid and ferric chloride (cf. LANGE, *Planta*, iii, 1927, p. 181). Transfer from water to 1 per cent. aqueous tannic acid for ten minutes. Add 1 per cent. sodium salicylate to the tannic acid as a fungicide. Wash thoroughly in water and place in 3 per cent. aqueous $FeCl_3$ several minutes. Cell-walls of meristems should appear black or dark blue, and nuclei and cytoplasm grey. Wash, and if too weak, replace in tannic acid solution. Transfer to 50 per cent. alcohol. Stain forty-eight hours in 1 per cent. safranin in 50 per cent. alcohol. Wash in 50 per cent. alcohol and destain in weak acid alcohol. Dehydrate and clear. NORTHERN (*Stain Tech.*, xi, 1936, p. 23) gives alternative schedules.

CYTOLOGICAL STAINS

1310. Most important are Heidenhain's, Mayer's and Cole's hæmatoxylins, Newton's gentian violet iodine (modified Gram stain) and thionin. Cole's rapid hæmatoxylin method is highly adaptable.

Stain combinations are best avoided for most work, as they are frequently capricious. But good results can frequently be attained. The following are the more important combinations used.

1311. Iron Hæmatoxylin and Safranin. See LENOIR (*Rev. Gén. Bot.*, xxxviii, 1926, p. 354). Mordant sections two to three hours in 3 per cent. ferric alum, wash in water and stain three hours in hæmatoxylin. Wash in water and differentiate in

3 per cent. alum. Stain in safranin for twelve to fifteen hours. Differentiate in weakly acidulated 70 per cent. alcohol until the cytoplasm loses its rose tint.

1312. **Safranin and Crystal (or Gentian) Violet** (HERMANN, *Arch. mikr. Anat.*, xxxiv, p. 58). Stain three to twenty-four hours in safranin (1 per cent. in 50 per cent. alcohol, made with anilin water); destain five to thirty seconds in 50 per cent. alcohol. Counterstain in a solution of crystal violet in anilin water for ten seconds to a few minutes. Dehydrate rapidly through 50, 70 and 95 per cent. alcohols, omitting 50 and 70 per cent. if stain lost too rapidly. Clear and differentiate in clove oil; wash in several changes of xylol.

Chromosomes and nucleoli red; resting nuclei and chromatin granules violet; prophase and early telophase nuclei may show violet chromonema and red chromatin granules; cytoplasm light violet and spindle deeper violet.

Substitution of dilute solution of crystal violet in clove oil for aqueous solution gives brilliant staining of nucleoli and chromosomes. Remove excess stain with clove oil.

The staining may be reversed in effect. Stain six to twenty-four hours in a 1 per cent. solution of crystal violet in 70 per cent. alcohol and destain briefly with 50 per cent. alcohol. Counterstain with safranin for fifteen seconds to five minutes. Dehydrate rapidly, clear in clove oil and wash in xylol. Chromosomes and nucleoli violet, cytoplasm and spindle red. See also STOCKWELL (*Science*, lxxx, 1934, p. 121) for a safranin, gentian violet staining method for difficult cases.

FLEMMING'S **Triple Stain.** See §§ 322 and 323.

1313. NEBEL'S **Crystal Violet and Acid Fuchsin** (*Zeits. Zellf. Mikr. Anat.*, xvi, 1932, p. 251). Stain from water in acid fuchsin and differentiate in picric acid. Stain in crystal violet and dehydrate rapidly.

1314. **Methyl Green and Acid Fuchsin** (GUIGNARD, *Rev. Gén. Bot.*, i, p. 11). Stain several hours in aqueous methyl green and differentiate in water or 50 per cent. alcohol until the stain is removed except from the nuclei and chromosomes. Stain for a short time (thirty seconds to three minutes) in aqueous acid fuchsin which tends to replace the methyl green. Dehydrate with absolute alcohol, clear in clove oil, wash in xylol and mount. Chromosomes and nucleoli green, spindle and cytoplasm pink.

1315. **Cyanin and Erythrosin.** Stain a few minutes to an hour or more in solution of cyanin in 70 per cent. alcohol. Rinse in 70 per cent. alcohol and stain a few seconds in erythrosin solution in 70 per cent. alcohol. Dehydrate rapidly with 95 per cent. and absolute alcohol. Clear in clove oil, wash in xylol and mount. Chromosomes blue, cytoplasm pink. If stains lost too readily omit 95 per cent. alcohol and clove oil.

1316. Breinl's **Triple Stain** (safranin, polychrome methylen blue and orange tannin). Mordant fifteen minutes in alcoholic iodine-potassium iodide solution, rinse with water and stain thirty minutes or longer in safranin (alcoholic solution). Wash with water, place ten minutes in polychrome methylen blue and again wash with water. Flood with orange tannin. When the orange has replaced the blue in the cytoplasm, wash well with 95 per cent. alcohol, rinse in absolute alcohol and clear with anilin oil, which also completes the differentiation. The action is not given by pure anilin oil, but by virtue of some impurity in the commercial product. Rinse with cedar oil, and mount in balsam. Chromosome threads blue, metaphase chromosomes red, cytoplasm pale yellow.

CHAPTER XLIX

SOME METHODS OF SPECIAL STAINING VITAL STAINING AND CYTOPLASMIC STRUCTURES

1317. Living Material. Relatively few plant structures (*e.g* unicellular and filamentous plants, pollen mother-cells, hairs, stripped epidermis) are adapted to intravital observation.

1318. Coloured cell structures (chloroplasts, chromatophores, eyespots) are best examined in the living state and require no special treatment. Chromatophores with faint pigmentation are made more evident by a suitable screen placed below the condenser (see Plastids).

For methods of investigating living cells of cambium see Bailey (*Zeits. Zellforsch. Mikr. Anat.*, x, 1930, p. 651) and Priestley, Scott and Malins (*Proc. Leeds Phil. and Lit. Soc.*, ii, 1933, p. 365). Bailey kept cambial initials alive and actively streaming for 500 hours in saccharine solutions and "white Russian oil."

Martens (*Bull. d'Histol. Appl.*, v, 1928, p. 229) studies the resting nucleus of living cells during the process of fixation.

See also Favorskii (*Mem. Soc. Nat. Kieff.*, xxvii, 1926, p. 71) for the so-called chromolytic method of studying living cells.

Dark Ground Illumination. See Price, *Ann. Bot.*, xxviii, 1914, p. 601; Strangeways and Canti, *Quart. J. Micr. Sci.*, lxxi, 1927, p. 1.

1319. Micro-culture Chambers. Kirchner (*Die mikroskopische Pflanzenwelt des Süsswassers*, 1885, Braunschweig) and Vosseler (*Zeits. Wiss. Mikr.*, vii, 1890, p. 457) use a square coverslip supported on a slide at its corners by small wax feet; the wax is made by adding to melted wax half to one-third its bulk of Venetian turpentine, stirring constantly.

1320. Hanging-drop methods consist essentially in a coverslip, with a drop of a suspension of the material on its lower side, supported on a wall built up on the slide; a fair volume of air is thereby provided. The van Tieghem cell is the best, and consists of a short piece of wide glass tubing cemented to the slide with balsam.

See also Farr (*Science*, lvi, 1922, p. 227), Murneek and Yocum (*Plant Physiol.*, ii, 1927, p. 506).

1321. Continuous Flow (Siphon) Chambers. Klercker (*Untersuch. bot. Inst. Tübingen*, ii, p. 333) uses a chamber prepared by cementing two strips of glass (about 0·15 mm. thick) to a slide

leaving a channel between them. The material is placed in the channel and covered with a coverslip; the space is then completely filled with liquid. A strip of linen is then pushed under the cover from each side and rubber bands or clips are used to fasten the coverslip to the slide. The linen strips are connected to a siphon system, one acting as inlet and the other as outlet.

GAUTHERET (*C. R. Acad. Sci. Paris*, cxcviii, 1934, p. 2195) grows explants of cambium and phloem on cotton saturated with culture media.

See also RHUMBLER, *Zeits. Wiss. Zool.*, xlvi., 1888, p. 549; SCHÖNFELD, *J. Roy. Micr. Soc.*, 1888, p. 1028; MCCORMICK, *Science*, lxxiii, 1931, p. 131.

See also under Fungi, methods of Vernon, Bachmann, Cifferi.

1322. Pollen Mother-cells. KUWADA and SAKAMURA (*Protoplasma*, i, 1926, p. 239) find that the chromosomes in pollen-mother cells of *Tradescantia* generally cannot be seen in cane sugar. This is the result of over-swelling. The *p*H especially has a striking effect on the distinctness of the chromosomes. They swell and finally lose visibility as the *p*H rises from 1·7 to 6·7. The swelling is reversible within wide limits. For *Tradescantia* the best *p*H is 5·0, or a little less; the medium requires to be well buffered, since the anther contents are alkaline and readily affect a lightly buffered solution (see also § 1374).

SAKAMURA (*Protoplasma*, i, 1926, p. 537) made observations on cells mounted in the viscous contents of the anther or in olive oil. Both media are superior to sugar and salt solutions. When mounted in olive oil, very few pollen mother-cells actually come into contact with the oil; the remainder are surrounded by contents of the anther even when this is small in amount.

SCHAEDE (*Ber. Deutsch. bot. Ges.*, xlviii, 1930, p. 342) mounted living staminal hairs of *Tradescantia* in liquid paraffin. See also SCHAEDE, *Protoplasma*, iii, 1928, p. 145.

1323. Intravital staining is limited in its application to plant material largely because the firm nature of the cell-wall impedes the entry of the dye. Further, cutin may prevent wetting of the material and the presence of pigments obscure the effect of the dye. Suitable material includes: fresh-water algæ; fungi; hairs of pistils, stamens, leaves, etc.; stripped epidermis; root-hairs; portions of aquatic plants. Such material can frequently be kept in good condition and stained in an isotonic solution. Suitable stains are Bismark brown in dilution of 1 : 3000, methylen blue and neutral red in dilutions of 1 : 1000 to 1 : 100,000. Such solutions stain the nuclei, plastids, etc., and the stain should become more intense as the cells become moribund.

See SANDS (*Science*, lvi, 1922, p. 517) for vital staining of *Tradescantia* chromosomes.

Neutral red penetrates more rapidly and is less toxic than other

vital stains. Becker (*Acta Soc. Bot. Poloniæ* vi, 1929, p. 214) found solutions of neutral red, at dilutions of 1 : 20,000 to 1 : 15,000, did not disturb the course of mitosis in *Stratiotes*, *Hydrocharis* and *Nuphar*. Compare also Becker, *Cytologia* iv,, 1933, p. 135, where it is shown that the appearance of abnormal mitoses depends upon the concentration and length of exposure to the dye. Guilliermond (*Bull. Hist. Appl. Physiol. et Path.*, iv, 1927, p. 125) finds that it stains only the contents of the vacuome and does not modify the structure. Hitchcock (*Bull. Torrey Bot. Club*, xlvi, 1919, p. 375) finds a differential staining of the cytoplasm of Characeæ by neutral red. Guilliermond has also made considerable use of Janus green B.

Küster (*Ber. Oberhessisch. Ges. Natur.-u. Heilkunde. Naturwiss.*, xi, 1927, p. 8) found bromo-phenol blue (cells coloured blue) and phenol red (cells coloured a deep yellow) were *p*H indicators unusually harmless to cells of the epidermis of *Allium* bulb-scales. He also found malachite green a vital stain in 0·1 per cent. solution. According to Albach (*Zeits. Wiss. Mikr.*, xliv., 1927, p. 333; *Protoplasma* v, 1928, p. 410) such a solution gives a clear vital stain in one minute; a 0·001 per cent. solution requires a longer time, but in it the epidermal cells remain alive forty-eight hours. Albach states that methyl green is also a vital stain. Paltauf (*Sitzungsber. Akad. Wiss. Wien Math.-Naturw. Kl.*, Abt. I, cxxxvii, 1928, p. 691) finds erythrosin, eosin and dahlia violet stain the living nuclei of several tissues. The cell sap is not stained. The colour is always diffuse, and is greatly aided by nitrates of K, Mg, Ca and Na, $Al_2(SO_4)_3$, alcohol, ether and high temperature, but not by light. Dahlia violet stains well without the addition of salts. Chrysoidin and water blue stain the cytoplasm, but not the nucleus. Isotonic solutions of neutral red, methylen blue or brilliant cresyl blue deeply stain the vacuolar material of living tissues; the reaction in many cases is apparently due to the presence of phenolic compounds.

See also Bailey and Zirkle, *J. Gen. Phys.*, xiv, 1931; Sherwood, *Proc. Exp. Biol. Med.*, xxiii, 1926, p. 622.

1324. Direct staining is frequently used on material without previous fixing and mordanting processes. The dyes are usually highly toxic. Iodine, eosin, picro-nigrosin, aceto-methyl green (see § 363) and aceto-carmine are most useful. Stain five minutes in 1 per cent. aqueous eosin, differentiate in water and fix the stain with 2 per cent. aqueous acetic acid. The material can be treated with aqueous iodine solution first to stain the starch and afterwards stained with eosin.

1325. Plasmodesmen (protoplasmic connections) are generally made more evident by swelling of the cell-walls. Swell with 25 per cent., or stronger, sulphuric acid to which iodine has been added. Or fix with 1 per cent. osmic acid and stain with crystal

violet. Material dehydrated and imbedded in the usual way is unsatisfactory. Intra-vitam staining is also unpromising.

Craft's method (*Stain Tech.*, vi, 1931, p. 127). Cut free-hand sections from fresh material and place them in a solution of 0·75 grm. KI and 0·5 grm. iodine in 100 c.c. water for five minutes. Swell five minutes with 10 per cent. sulphuric acid. Then mordant five minutes in a solution of 1 grm. iodine and 1·25 grm. KI in 100 c.c. of 5 per cent. sulphuric acid. Wash in 5 per cent. sulphuric acid until the iodine starts to fade, changing the liquid once or twice. Transfer to freshly mixed stain (0·5 per cent. aqueous gentian violet in 5 per cent. sulphuric acid, made up to form a dark green solution). Heat to about 50° C. and allow to stand five minutes. Wash sections quickly in 5 per cent. sulphuric acid. Intensify the stain and clear the cell walls by immersion in a weak solution of iodine-potassium iodide in 5 per cent. sulphuric acid for one to two minutes. Mount in a mixture of 30 c.c. glycerin, 2 grm. zinc chloride and 0·1 grm. iodine and a bit of KI in 60 c.c. water. Eventually the stain crystallises out; keep the preparation cool to retain it longer. See also Livingston, *Amer. J. Bot.*, xxii, 1935, p. 75.

1326. Spermatozoids, zoospores, motile gametes and other motile naked bodies are best fixed as a suspension in a drop of water (or culture medium) with the vapour from a 1 per cent. osmic acid solution (Steil, *Bot. Gaz.*, lxv, 1918, p. 562). Dry the slide and stain. A variety of stains may be used. Steil stains in safranin ten minutes to one hour, then washes in water and in 95 per cent. alcohol until only the nucleus remains stained. If necessary, next clear in xylol and remove the xylol with 95 per cent. and absolute alcohol. Then stain in acid fuchsin ten to twenty seconds, wash in absolute alcohol, clear in clove oil and xylol and mount in balsam. Nucleus bright red, cytoplasm a bluish-pink. He also obtained good results by staining in iron hæmatoxylin. Iodine green and acid fuchsin, Delafield's hæmatoxylin, Flemming's triple and others may also be used.

Showalter (*Ann. Bot.*, xl, 1926, p. 702) considers Heidenhain's hæmatoxylin best for details of Bryophyte antherozoids. He recommends Bismark brown as a counterstain to show the gelatinous envelope. For the general form, and some details, stain in dilute gentian violet, rinse in water, dry, and add a drop of balsam and a coverslip.

For Cotner's method for fungal zoospores see § 1399.

Cilia. To observe, mount organisms in a dilute solution of cocaine. They may be observed in motion by the method of Bütschli (*Ueber de Bau der Backterien und verwandter Organismen*, Leipzig, 1890). He suspends fine granules of carmine in the fluid containing the organisms.

Migula (*Bot. Centrbl.*, xliv, 1890, p. 72) adds a very small

drop of concentrated alcoholic solution of cyanin to living material and, after a time, enough water to precipitate the cyanin not taken up. The cilia are at first pale blue, but after the addition of water deep violet.

Rapid killing with osmic acid vapour, 1 per cent. osmic acid, 1 per cent. chromic acid or iodine-potassium iodide solution is essential.

Zimmermann fixes a drop of suspension on a slide with osmic vapour and allows it to dry. Add a drop of 20 per cent. aqueous tannin and wash off with water after five minutes. Stain in concentrated aqueous fuchsin, or, better, carbol-fuchsin. Wash with water, dry and mount in balsam. Loeffler's flagella stain (for bacteria) can be used for difficult cases. See also HOLLANDE, *Arch. zool. exp. et gén.*, lix, 1920, p. 75.

For algal and fungal zoospores add a drop or two of 1 per cent. osmic acid to the water containing them and then a drop or two of strong alcoholic solution of equal parts of fuchsin and methyl violet.

1327. Nucleoli. Zimmermann recommends fixation with concentrated alcoholic solution of corrosive sublimate and staining by Altmann's acid fuchsin-alcoholic picric (*Arch. Anat. Physiol., Anat. Abt.*, 1889, p. 409), Zimmermann's acid fuchsin and acid fuchsin-potassium bichromate methods or double staining with acid fuchsin and Delafield's hæmatoxylin.

LENOIR (*C. R. Soc. Biol.*, cix, 1932, p. 471) fixes *Equisetum* sporangia in a formalin-acetic acid-chromium acetate mixture, mordants in alcohol and HCl, stains with carbolated fuchsin D and differentiates with a mixture of clove oil, absolute alcohol and picric acid. The nucleolin is intensely stained to the exclusion of reticular chromatin.

LENOIR (*C. R. Soc. Biol.*, cxviii, 1935, p. 1554) also recommends fixation in Bouin-Duboscq-Brasil (LENOIR, *C. R. Soc. Biol.*, ciii, 1930, p. 1253), followed by double staining with basic fuchsin and malachite green (LENOIR, *C. R. Soc. Biol.*, civ, 1930, p. 1282). The chromosomes are red, the nucleolus green. The stain fades in time.

DANNEHL and ZIEGENSPECK (*Bot. Archiv.*, xxv, 1929, p. 243) used triacid staining for distinguishing nucleoli from chromosomes at all stages.

See also § 646 and ZIRKLE, *Bot. Gaz.*, lxxxvi, 1928, p. 402.

1328. Other Cell Organs. Centrosomes and blepharoplasts are very responsive to osmic acid, failing to be visible following chrom-acetic fixation without it. Polar caps and spindle fibres are less distinct following fixatives where the osmic is large in amount. The osmic acid should be reduced as far as possible in Flemming fluids used for this purpose, but if too little in amount the cytoplasm appears as a mass of coarse vacuoles.

Schaede (*Beitr. Biol. Pflanzen*, xiv, p. 367) has a modification of Juel's fixative to show spindle structure.

1329. Cytoplasmic Inclusions. Plant cytoplasm appears to contain three systems of self-perpetuating structures, viz. mitochondria, plastids and their primordia, and osmiophilic platelets (probably equivalent to the Golgi of animal cells). The majority of the methods used in their study are identical with those used on animal tissues. The behaviour of these methods, however, is more variable with plant material. Bowen stresses the use of fresh material, of freshly mixed solutions, of glass-stoppered bottles and very careful handling of osmicated material. Some at least of the variability of osmium impregnation follows from different conditions of growth of the plant material. Thus Bowen and Buck (*Ann. Bot.*, xliv, 1930, p. 565) find Benda's stain is best on sand-culture material, while the Kull stain is more indifferent.

The identity of the plant equivalent of the Golgi apparatus is still a matter of controversy. Bowen (*Anat. Rec.*, xxxiv, 1926, p. 143; *ibid.*, xxxv, 1927, p. 309; *Biol. Bull.*, liii, 1927, p. 179; *Zeits. Zellf. Mikr. Anat.*, vi, 1928, p. 689; *ibid.*, ix, 1929, p. 1; *Ann. Bot.*, xliii, 1929, p. 309; *Bull. Torr. Bot. Club*, lvi, 1929, p. 33) and Gatenby (*Nature*, cxxi, 1928, p. 11; *Proc. Roy. Soc.* B civ, 1929, p. 302; *J. Roy. Micr. Soc.*, III, l, 1930, p. 20) favour the osmiophilic platelets, which are best seen by the Kolatchev method. Weier (*Proc. Nat. Acad. Sci.*, xvi, 1930, p. 536; *La Cellule*, l, 1931, p. 261; *ibid.*, li, 1931, p. 51; *Biol. Bull.*, lxii, 1932, p. 126; *Amer. J. Bot.*, xix, 1932, p. 659) finds that moss plastids, following osmium and silver impregnations as used by Parat and others, show images similar to those of the Golgi apparatus.

The "canaliculi" of Bensley (*Biol. Bull.*, xix, 1910, p. 174), alleged to be the primordia of the vacuoles and to resemble the Golgi of animal cells, were obtained chiefly by using neutral formalin-potassium bichromate fixatives. Guilliermond and Mangenot (*C. R. Acad. Sci.*, clxxiv, 1922, p. 692) blackened the same, or similar, structures by silver impregnation methods. See also Guilliermond, Mangenot and Plantefol (*Traité de Cytologie Végétale*, 1933, Paris) for numerous references.

Bowen finds the "vacuome" is most regularly blackened by the Weigl method, but the manner of impregnation is very variable. In fact Patten, Scott and Gatenby (*Quart. J. Micr. Sci.*, lxxii, 1928, p. 387) consider the vacuome of Bowen may be an artifact, caused by the variability of the water to wash out the corrosive sublimate of Mann's fluid, previous to osmification.

See also Concalves de Cunha, *C. R. Soc. Biol.*, Paris, xcix, 1928, p. 1538.

A further complication is introduced by the presence of proplastids (plastid-primordia), which resemble and have been identi-

fied by some with mitochondria. Zirkle (*Bot. Gaz.*, lxxxviii, 1929, p. 186) defines as mitochondria all small inclusions preserved by bichromates with a *p*H greater than 4·2 to 5·2 (dependent on the cation) and destroyed by more acid bichromates and by mixtures of bichromates and acetates; he restricts the term "plastid" to those bodies containing starch or chlorophyll. He fixes plastids and their primordia in 1·25 grm. potassium bichromate, 1·25 grm. ammonium bichromate, 1 grm. copper sulphate and 100 c.c. water.

The mitochondria and the pro-plastids appear to react differently to impregnation (see Bowen, *Zeits. Zellf. Mikr. Anat.*, vi, 1929, p. 689) and have a different appearance and behaviour in tissues affected with mosaic (Dufrénoy, *Rev. Path. Vég. Entom. Agr.*, xviii, 1931, p. 74). But Ozawa (*Rev. Gén. Bot.*, xxxix, 1927, p. 218) finds that plastids, proplastids and mitochondria react similarly.

The mitochondria are best seen by Champy-Kull, the plastids by Weigl and in Benda preparations.

A few special methods and modifications of animal methods have been suggested.

1330. Mitochondria are preserved by neutral formalin; fix forty-eight hours, wash in water and stain in Heidenhain's hæmatoxylin. Mottier (*Ann. Bot.*, xxxii, 1918, p. 91) finds a mixture of 17 c.c. 1 per cent. chromic acid, 3 c.c. 2 per cent. osmic acid and 3 drops acetic acid the best fixative.

See also Zirkle, *Science*, lxvi, 1927, p. 400.

Ozawa (*Rev. Gén. Bot.*, xxxix, 1927, p. 218) states that Regaud (without post-chroming), Tupa and Helly are most satisfactory, and that all other mitochondrial fixatives, especially those containing osmic, produce marked alterations. He used the bud of *Elodea canadensis*. According to Bowen, the Champy-Kull method is practically specific. Levitsky (*Ber. Deuts. bot. Ges.*, xxviii, 1910, p. 540) used a mixture of 8·5 parts of 10 per cent. formalin and 1·5 parts of 1 per cent. chromic acid, followed by treatment with Benda. The formalin-chromic alone, was less satisfactory since staining became more difficult.

Lenoir (*Rev. Gén. Bot.*, xxxviii, 1926, p. 720) uses a mixture of equal parts of 2 per cent. KI and 2 per cent. chromic acid. This rapidly fixes and stains the mitochondria in epidermal cells of *Allium cepa*.

Mascré (*C. R. Acad. Sci.*, clxxxv, 1927, p. 866; clxxxviii, 1929, p. 811) states that a preliminary treatment with formaldehyde protects the mitochondria from the solvent action of acetic acid. He finds that monochloracetic, trichloracetic and cyanacetic acids fix the mitochondria, but treatment with formalin is essential to prevent subsequent solution in the rinse water. He uses the following mixtures:—

20 per cent. formalin and 3 per cent. monochloracetic acid (pH 1·6).

20 per cent. formalin and 3 per cent. cyanacetic acid (pH 1·3).

20 per cent. formalin and 2 per cent. trichloracetic acid (pH 0·6).

However, a mixture of 20 per cent. formalin and 5 per cent. acetic acid (pH 1·9) does not fix the mitochondria. After fixation, wash rapidly in water, treat six hours with iron alum, stain twelve hours with hæmatoxylin and differentiate with iron alum.

KASSMANN (*Planta*, i, 1926, p. 624) describes, for the purpose of studying mitochondria and plastids, a technique for permitting given cells in living shoots of *Cabomba* to be kept under continuous observation for months.

GUILLIERMOND (*C. R. Acad. Sci.*, clxx, 1920, p. 1329) recommends neutral red and cresyl blue for vitally staining the mitochondria. The latter stain differentiates the metachromatic bodies from the mitochondria. He also (*C. R. Soc. Biol.*, lxxxix, 1923, p. 527) uses dahlia violet and Janus green. The plastids colour more slowly and less easily than the mitochondria. Aquatic fungi, such as *Saprolegnia*, are more adaptable to intra-vitam staining than are tissues from higher plants, the cell walls of which are less permeable to the dyes.

1331. Double Staining of Mitochondria and Starch Grains. Guilliermond recommends iron-alum hæmatoxylin and iodine, osmic acid and iodine, or acid fuchsin, toluidine blue and aurantia (Kull's stain, § 695).

MILOVIDOV (*Arch. Anat. Microsc.*, xxiv, 1928, p. 9) describes adaptations of Volkonsky's methods.

(1) Fix in Regaud or chromo-formol-bichromate. Stain in acid rubin for five minutes at 60 to 80° C., differentiate under the microscope in 5 per cent. alcoholic aurantia and wash in water. Mordant twenty minutes in 2 per cent. aqueous tannin solutions and wash in water. Stain five to ten minutes in 1 per cent. aqueous toluidine blue or methyl green or gentian violet, differentiate in alcohol, dehydrate, clear and mount. Mitochondria red, starch grains blue, green or violet.

(2) Fix in Meves, Regaud or chromo-formol-bichromate, mordant in 3 per cent. iron alum and stain in hæmatoxylin for twenty-four hours; differentiate in iron alum and wash well. Mordant in 2 per cent. aqueous tannin for thirty to sixty minutes and wash in distilled water. Then mordant in 1·5 per cent. tartar emetic and wash in water. Stain in 1 per cent. aqueous gentian violet thirty to sixty minutes, differentiate in alcohol, dehydrate, clear and mount.

1332. Double Staining of Bacteria and Mitochondria in Plant Tissues. The methods are intended for the root nodules of Leguminosæ, etc., the leaf tips of *Dioscorea* and for other tissues containing symbiotic or parasitic bacteria.

Milovidov (*Arch. Anat. Microsc.*, xxiv, 1928, p. 19) fixes in chromic-formalin or Neměc's chromic-bichromate-formalin and treats with Volkonsky's modification of Kull's method. Stain in 20 per cent. acid rubin in anilin water for five minutes at 60 to 80° C. After cooling wash in distilled water, differentiate in 5 per cent. aurantia in 70 per cent. alcohol and wash quickly in water. Treat for two to three minutes with a solution of 10 c.c. normal caustic soda and 1 grm. phosphomolybdic acid in 90 c.c. distilled water and wash quickly in water. Then stain ten to fifteen minutes in a solution of 50 c.c. glycerin, 0·1 grm. potassium carbonate, 0·1 grm. azur II and 0·4 grm. methylen blue in 50 c.c. distilled water. Wash in water, differentiate in Unna's tannin-orange, wash in water, dehydrate and mount.

Milovidov (*C. R. Soc. Biol.*, xcviii, 1928, p. 555) and Dufrenoy (*Stain Tech.*, iv, 1929, p. 13) give the following schedule: Fix in a mixture of 1 per cent. chromic acid 50 c.c., 1 per cent. potassium dichromate 50 c.c. and 40 per cent. neutral formalin 8 c.c., rinse twenty-four hours in running water, dehydrate, imbed and cut at 4μ. Dissolve the paraffin from the slides, dip in a very thin solution of collodion in absolute alcohol and ether and run through the alcohols to water. Stain in anilin-acid fuchsin and wash in water. Destain in aurantia in 70 per cent. alcohol and wash in water. Treat for a few minutes with 1 per cent. phosphomolybdenic acid in 0·1 per cent. caustic soda, rinse and stain in Unna's polychrome methylen blue. There results a very sharp differentiation of the bacteria which are stained a deep violet blue, while the mitochondria and plastids retain the red of the acid fuchsin.

1333. Plastids. Most standard fixatives yield little or no information on their structure, though some, especially of the Flemming group, are suitable for studies of their form. Zimmermann recommends a saturated solution of picric acid and corrosive sublimate in absolute alcohol. Krasser (*Bot. Centrbl.* lii, 1892, p. 4) uses a 1 per cent. alcoholic solution of salicylic aldehyde. Zimmermann recommends staining with acid fuchsin (Altmann or other methods), iodine green or ammoniacal or basic fuchsin. The latter two stains will not withstand dehydration through alcohol and are best examined at once in glycerin. These methods also suffice for pyrenoids. Zirkle (*Amer. J. Bot.*, xiii, 1926, p. 301) studied the distribution of pigments in the chloroplast by means of monochromatic light of wavelengths corresponding to the absorption and bright bands of the several pigments. He also employed frozen sections (not below − 4° C.) and chloroplasts extruded from the cell into a culture medium of lactose, gelatin and glycin.

Lowe and Lloyd (*Trans. Roy. Soc. Canada*, III, xxi, 1928, p. 279) demonstrated the chloroplast in *Hydrodictyon* by the use of light in the region of the absorption bands of chlorophyll.

MAIGE (*C. R. Soc. Biol.*, xcv, 1926, p. 299) studied amyloplasts gradually turning green.

1334. Pro-plastids. Potassium bichromate fixatives cause them to elongate. Acetic acid destroys them; later they become resistant to this reagent. After osmification and silver impregnation they appear hollow with a blackened periphery. See KIYOHARA, *Bot. Mag.*, Tokyo, xli, 1927, p. 211; *Cytologia*, i, 1930, p. 328; WEIER, *La Cellule*, xl, 1931, p. 261; ZIRKLE, *Amer. J. Bot.*, xiv, 1927, p. 429. They form a system distinct from the mitochrondia and are best seen in Benda fixed preparations.

1335. Pyrenoids show after Flemming and other common fixatives. In material fixed in saturated alcoholic $HgCl_2$, washed thoroughly and stained twenty-four hours in a 0·2 per cent. solution of acid fuchsin, the pyrenoids are bright pink and the nucleoli unstained. (See also § 1333.)

1336. Elæioplasts may be fixed like plastids. See WAKKER (*Jahrb. f. Wiss. Bot.*, xix, 1888, p. 423). Fix in saturated aqueous picric acid and stain in an aqueous solution of anilin blue that has been turned purple by the cautious addition of alkanin. After several hours the elaieoplasts show purple, cytoplasm light blue, nuclei dark blue, oil droplets red. The colours last for a long time in neutral glycerin jelly.

CHAPTER L

MICROCHEMICAL TESTS

1337. In this chapter are given some tests for microchemical substances as applied to plants. Additional information will be found in Chapter XLVII and Chapters XXVII, XXVIII and XXIX.

INORGANIC

Studies of the distribution of soluble, diffusible salts are best made on sections prepared by a freezing method. See § 1340.

1338. **Sulphur** may be found in the free state as globules on or in filaments of algæ, bacteria and fungi growing in sulphurous water. The globules are soluble in CS_2 if the plants are first killed by drying or otherwise. See also Klein, *Oesterr. Bot. Zeits.*, lxvi, 1927, p. 15.

1339. **Silica** is best recognised after incineration of sections, the siliceous portions remaining comparatively unaltered. It is soluble in HF. For silica in the cell-wall, Molisch treats sections on a slide, coated with varnish or Canada balsam, with aqua regia or Schultze's reagent, washes with water and then tests with HF. See also Brown, *Bull. Torrey Bot. Club*, xlvii, 1920, p. 407.

1340. **Potassium.** Macallum (*Journ. Physiol.*, xxxii, 1905, p. 95) uses the hexanitrate of Co and Na, which in the presence of sodium acetate gives an immediate precipitate for potassium. The precipitate is rendered more visible by ammonium sulphide, which makes it densely black. Dowding (*Ann. Bot.*, xxxix, 1925, p. 459) freezes the material with solid CO_2 at $-50°$ C., cuts it frozen and thaws it after it is placed in the reagent. This prevents diffusion. Place sections one to two minutes in the cobalt reagent, wash twenty to thirty minutes in a succession of basins kept over ice-cold water over a freezing bath and mount in glycerine and fresh ammonium sulphide.

According to Molisch (*Microchemie der Pflanzen*, lx, 1921) ammonia gives a similar reaction, but its presence in fresh material is unlikely. Nessler's reagent will test its presence.

1341. **Sodium.** Streng (*Zeits. Wiss. Mikr.*, iii, p. 129) recommends the use of uranyl-magnesium acetate. Schimper (*Flora*, 1890, p. 207) used uranyl acetate. Molitylin and Tubokario (*Microchem.*, vii, 1929, p. 334) use zinc uranyl acetate.

1342. **Calcium** in the cell sap may be recognised by the addition of ammonium oxalate or ammonium carbonate, forming calcium

oxalate or carbonate crystals respectively. As the pectate in the middle lamella, it is best recognised by the addition of pure sulphuric acid; needle-form crystals appear in a few minutes (Molisch).

BAECKER (*Mikrokosmos*, Stuttgart, xxiii, 1930, p. 126) fixes material in a Ca-free mixture and sections it. If imbedded, care must be taken that the reagents are Ca-free. Tests: (1) Float sections in 3 per cent. H_2SO_4 under a coverslip; needles of $CaSO_4$ form in a few minutes. (2) Stain five to ten minutes in saturated alcoholic purpurin solution, treat a few minutes in 0·75 per cent. (physiologic) salt solution, destain in 70 per cent. alcohol and mount in balsam. Calcified structures, or (if decalcified) structures formerly containing calcium, are stained an intense rose colour. (3) Treat sections half to one hour in 5 per cent. aqueous solution of $AgNO_3$ in bright light; wash in distilled water and treat one to two minutes with $\frac{1}{2}$ to 1 per cent. aqueous pyrogallol and then fix five minutes in 5 per cent. sodium thiosulphate. Calcified areas appear black.

1343. Magnesium. SCHIMPER (*Flora*, 1890, p. 214) recommends the addition of a solution of sodium phosphate or of microcosmic salt ($NaNH_4HPO_4$) reduced with a little ammonium chloride. Rhombic crystals of ammonio-magnesium phosphate ($MgNH_4PO_4$) are formed. See also KLEIN, *Oesterr. Bot. Zeits.*, lxvi, 1927, p. 15.

1344. Iron. See § 674.

1345. Phosphorus. See ANGELI, *Riv. Biol.*, x, 1928, p. 702.

ORGANIC

1346. Fats. These are treated fully in Chapter XXVIII, page 278, to which refer.

Fats are immiscible with water and have a different refractive index; their microscopic appearance in aqueous mounts is therefore characteristic. They are soluble in ether, chloroform, benzene, etc. Fats are fairly rapidly turned brown and then black by 1 per cent. osmic acid solution, but osmic acid also stains proteins brown. Moreover, saturated fats and fatty acids sometimes do not reduce osmic acid. They are stained by alkannin, Scharlack R (HILL, *New Phyt.*, xi, 1912, p. 72) and Sudan III, but these and similar reactions are not specific. RANVIER (*Tech. Lehrb. Hist.*, Leipzig, 1888) uses an alcoholic solution of cyanin.

ZWEIBAUM and MANGENOT (*C. R. Soc. Biol.*, lxxxix, 1923, p. 540) find that indophenol blue obtained in nascent state by a mixture of α-naphthol and dimethyl-paraphenylene-diamene (the "nadi" mixture) is a good vital stain for fatty substances. Fats stain a deep blue, essential oils a violet-rose, suberin and cutin a deep violet and lignin a very pale blue.

Saponification of fats under the microscope was first carried out by Molisch (*Grundriss einer Histochemie der planzlichen Genussmittel Jena*,

1891). Place sections in a drop of a mixture of equal parts concentrated KOH solution and concentrated ammonia solution. After half an hour to one hour or more the oil-drops, which steadily become less refractive, harden into myelin-like or botryoidal bodies or into irregular masses (soaps) often consisting wholly of small crystal-needles. See also ECKERSON, *Fats* (McCLUNG'S *Micr. Tech.*, p. 160); MICHAELIS, *Fat* (KRAUSE'S *Encyklopädie Mikr. Tech.*, Berlin, 1926); CIACCIO, *Pathologica*, xiii, 1921, p. 183; CZAPEK, *Ber. Deut. bot. Ges.*, xxxvii, 1919 p. 207 (lipoids).

1347. Waxes. See DE BARY (*Bot. Zeitg.*, 1871, p. 132). They are insoluble in water and melt together into drops in hot water. Insoluble or nearly so in cold alcohol, soluble in hot alcohol. On heating in a solution of alkannin in 50 per cent. alcohol, they run together into red drops.

1348. Sugars. MOLISCH (*Sitzungsber. Akad. Wiss. Wien.*, xciii, 1886, p. 912) finds the two following reactions common to many carbohydrates: (1) Add a drop of 15 to 20 per cent. alcoholic α-naphthol solution and then 2 to 3 drops concentrated sulphuric acid; a violet colour results. (2) Thymol used in the same way gives a carmine-red colour.

The reduction of copper salts by sugars in the presence of excess alkali is generally employed. But this reaction is unsatisfactory because of the considerable diffusion that occurs and because sucrose, if suspected, must first be hydrolysed by boiling with acid.

The best results have been secured by MANGHAM (*New Phyt.*, x, 1911, p. 160; *Ann. Bot.*, xxix, 1915, p. 369) and others using the osazone test first introduced by SENFT (*Sitz. Akad. Wiss.*, cxiii, 1904, p. 3; *Bot. Centrbl.*, 1904, p. 28). It is very delicate, giving glucose, for instance, in 0·015 per cent. solution. But it is comparatively slow. Two solutions are used: (1) 1 grm. phenylhydrazine hydrochloride in 10 grm. of glycerin, and (2) 1 grm. sodium acetate in 10 grm. glycerin. Aid the solution by heating, if necessary, and filter before use. Glycerin is used because it is more penetrating than water and also will not evaporate and deposit crystals of the substances used. It must be *pure*, for commercial glycerin is often adulterated with sugars. The glycerin retards crystal formation, the preparations therefore cannot be used for some time. Mix 1 drop of each solution on a slide, add a section (which must be at least one cell thick) and a coverslip. Heat on a water bath in a water-jacketed oven for about half an hour, allow to cool and seal with a mixture of gum mastic and paraffin wax; the osazone crystals will form in varying degrees of rapidity. Compare with the osazones given by solutions of known sugars.

Methylphenylhydrazine gives a crystalline osazone with levulose but not with dextrose.

1349. Starch. The most characteristic test is the blue reaction

with iodine. In the presence of KI or of hydriodic acid the colour is more violet-brown. The use of dilute solutions is always best, as the grains become almost black in concentrated solutions; further, the lamellæ show in dilute solution. Prepare an aqueous solution immediately before using by adding a few drops of an alcoholic solution to a few cubic centimetres of distilled water. Stain starch, in tissues, with 2 per cent. aqueous cotton red and counterstain the plastid with methylen blue.

Free starch grains are best stained with crystal (or gentian) violet (Němec, *Ber. Deut. Bot. Ges.*, xxiv, 1906, p. 528); after staining pour off the dye and wash with saturated aqueous picric acid. Dry and mount in balsam. Sections can be dehydrated and cleared in xylol. Safranin and thionin are also good stains. See also Kraemer, *Science*, lvii, 1923, p. 175; Dodge, *J. Appl. Micros.*, i, 1898, p. 99.

In polarised light, starch grains which are doubly refractive give a characteristic black cross, the centre of which corresponds with the hilum. See Sponsler (*Amer. J. Bot.*, ix, 1922, p. 471) for method of X-ray study of starch grains.

Glycogen (see also § 667) occurs in solution in fungal cells. It gives with iodine solution (0·1 grm. iodine, 0·3 grm. KI, 45 c.c. water) a red-brown colour which disappears temporarily on warming. It can be precipitated in the cell and, in fact, may be troublesome in fungi. Treat the material (*e.g.* fungal hyphæ) with absolute alcohol or Carnoy for twelve hours, and then with 10 per cent. tannic acid for twelve hours. Wash quickly and stain in ferric chloride solution; glycogen stains black.

For Best's carmine stain, see § 670. See also Mayer, *Zeits. Wiss. Mikr. mikr. Tech.*, xxvi, 1910, p. 513.

1350. Tannins occur in solution in vacuoles and give a blue-black or green colour with neutral solutions of iron salts. Use an ether solution of anhydrous ferric chloride (Moeller, *Ber. Deut. Bot. Ges.*, 1888, p. 66) or concentrated aqueous solution of ferrous sulphate (Loew and Bokorny, *Bot. Centrbl.*, xxxix, 1889, p. 369).

They can be precipitated and stained in the living state with aqueous methylen blue (1 : 500,000) (Pfeffer). Fix the stain with saturated aqueous picric acid for a few hours, rinse, dehydrate, clear in xylol and mount.

They reduce Fehling's solution, are precipitated by basic lead acetate and other metallic salts, and give a brownish precipitate with strong aqueous potassium bichromate or 1 per cent. chromic acid. A red-brown to brown colour is given by a sparing amount of a dilute ammoniacal solution of potassium ferricyanide; excess of the reagent destroys the colour. Aqueous iodine-potassium iodide solution, mixed with a little 10 per cent. ammonia produces a brilliant red colour.

GARDINER (*Proc. Camb. Phil. Soc.*, iv, 1883, p. 387) adds a solution of ammonium molybdate in a strong solution of ammonium chloride; many tannins give a copious yellow precipitate, while digallic acid gives a red coloration, destroyed by oxallic acid.

With gallic acid, KCN gives a pink coloration and Nessler's reagent a grey-green precipitate.

VINSON (*Bot. Gaz.*, xlix., 1910, p. 222) fixes and stains tannins *in situ* by precipitation with vapour of amyl or ethyl nitrate. Whole organs are exposed to the vapour. A 20 per cent. alcoholic solution made by diluting the 90 per cent. commercial nitrous ethyl is recommended. Amyl nitrite is disagreeable to use. Sweet spirits of nitre (containing about 4 per cent. ethyl nitrite) require a longer exposure.

1351. Proteins. Iodine gives a yellow to brown coloration, and osmic acid a brown coloration. A brick-red colour is obtained when they are warmed in a few drops of Millon's reagent. This reagent is a mixture of mercuric and mercurous nitrates and nitrous acid. PLUGGE (*Arch. Pharmacie*, ccxxviii, 1890, p. 44) dissolves 1 part by weight of mercury in 2 parts of nitric acid of s.g. 1·42 and then dilutes it with twice its volume of water.

Xanthoproteic Reaction. Warm with a few drops of strong nitric acid. A yellow colour, changed to orange by moistening with strong ammonia results. This is non-specific.

Biuret Reaction. Add a solution of copper hydrate in KOH solution; or steep twenty to sixty minutes in 0·2 per cent. aqueous KOH, wash and place in 10 per cent. copper sulphate for thirty to sixty minutes, wash in water and mount in 2 per cent. aqueous KOH. A mauve to violet colour indicates proteins. Protein stored in granular form is preserved by ordinary fixatives. Lyons blue gives an intense stain. Aleurone grains are well fixed by alcoholic mercuric chloride or picric acid. Stain the ground substance with alcoholic eosin; the globoid and crystalloid will show by contrast.

1352. Alkaloids. A chocolate brown precipitate is given by iodine-potassium iodide; colourless amorphous precipitates by tannic acid, phosphotungstic acid and mercuric iodide in potassium iodide; and crystalline precipitates by auric and platinic chlorides.

In examining plant tissues for alkaloids, ERRERA (*Ann. Soc. belge Micr. Mém.*, xiii) recommends testing fresh sections with alkaloid reagents and also sections that have been soaked with 5 per cent. alcoholic tartaric acid solution, which is a solvent of alkaloids. In the latter case, no precipitates should be obtained. The final identification of the various alkaloids depends chiefly upon the colour reactions, for which see various monographs.

See also NIETHAMMER, *Biochem. Zeits.*, ccxiii, 1929, p. 138.

Chaze (*Bull. d'Histol. Appl.*, v, 1928, p. 253), studying the formation of alkaloid in *Nicotiana* seedlings uses a combination of the two following methods: (1) Lightly press young radicles between coverslip and slide and treat with a dilute solution of neutral red. Aleurone grains in process of transformation into semi-fluid vacuoles are stained. (2) Use of Bouchardat's reagent, since aqueous neutral red tends to dissolve the nicotine.

1353. Nucleus (see also § 623). Shinke and Shigenaga (*Cytologia*, iv, 1933, p. 189) find that the chromosomes and nuclear "reticulum" show reactions for thymo-nucleic acid, lipoids and proteins. The reaction (Feulgen) is restricted to the spiral parts of the chromosomes, which are further dissolved by lipoid and nucleoprotein solvents. The karyolymph is a lipoid. The nucleolus contains lipoids, but no thymonucleic acid. The spindle and fibres and phragmoblast are mainly of proteins and lipoids in a mixed or combined form.

Margolena (*Stain Tech.*, vii, 1932, p. 9) has further examined the application of Feulgen's reaction to plant material. He finds that nucleoli never react except when the sections are specially treated. Lignin, suberin and cutin always react. Unhydrolised yeasts show a number of reddish granules, apparently due to simple oxidation and not to the presence of aldehydes. Hydrolised yeasts show a purplish nucleus.

The nuclei of some plants do not give the reaction. See also Neměc, *Ber. Deut. Bot. Ges.*, xxvii, 1909, p. 43; Kuwada and Sugimoto, *Protoplasma*, iii, 1928, p. 531; Westbrook, *Ann. Bot.*, xliv, 1930, p. 1011.

1354. Volutin. Fix with alcohol, formaldehyde or picric acid solution. Stain with fuchsin or methyl violet. Eosin and gentian violet-iodine (Gram's method) do not stain volutin. It darkens readily with iron-alum hæmatoxylin, but readily loses the stain on differentiation. It is best to stain with carbol-fuchsin and fix by treatment with aqueous I–KI solution.

General references, see Chapter XLVI, and Sampson, *Bot. Gaz.*, lxvi, 1918, p. 32; Brunswick, *Naturwiss.*, xi, 1923, p. 881; McCulloch, *Science*, lxxiv, 1931, p. 634 (apparatus for observation of a small object while flooded with various solutions); Czapeck, *Biochemie der Pflanzen*, Jena, 1920; Molisch, *Microchemie der Pflanzen*, Jena, 1913; Haas and Hill, *An Introduction to the Chemistry of Plant Products*, London, 1928.

CHAPTER LI

PLANT CHROMOSOMES †

1355. Many methods adapted to rapid chromosome counting, though useful for genetical purposes, are not suitable for exact studies of the nuclear cycle. But the line of demarcation is not sharp and numerous workers have used inferior methods for supposedly critical work. It is a common mistake to suppose that because cells appear unshrunken and the chromosomes distinct at metaphase, all other cell division stages (and cytoplasmic structures) must be correctly preserved.

Bělař (*Zeits. Ind. Abst. Vererb.*, Suppl. 1, 1928, p. 402), from a comparison of living and fixed material, finds that even the best technical methods produce more or less artificial changes in leptotene and interkinesis stages. It is unsafe to draw conclusions concerning the nuclear cycle from material fixed by the older methods, involving unopened or even opened flower buds, whole anthers and cells in depths of root-tips in higher plants, or comparable conditions in lower plants. Possibly there has been no really satisfactory fixation of many stages of mitosis in plants owing to the difficulty of securing immediate access of the fixative to the cells. Probably considerable success would attend fine chopping of root-tips under the surface of the fixative.

The nuclear structures are often well preserved although the cytoplasm is badly fixed. But the space relation between the formed elements is then often seriously disturbed and, unless the karyolymph is well and finely precipitated, the chromosomes tend to clump and contract. Exclusion of such artifacts is of great importance in studies of somatic pairing and of secondary pairing at meiosis in allopolyploids. Organic adjuvants, such as urea, various sugars and malic acid, assist in fixing the karyolymph, in preventing clumping, and in maintaining the spaces between longitudinally split chromosomes and between closely paired chromosomes in meiotic prophases.

The best standard of quality of a preparation is experience. In general, with a given material, greater ease of observations denotes a better preparation. Direct comparison with living cells can be made, but the technique of such observations and of those on material under weak intravital staining is very difficult; further, the basis of interpretation is different from that involved in the examination of material in resinous media.

The karyolymph should be coagulated as a fine granular soft mass, thereafter requiring gentle hardening in close grades of alcohol. The

† Read § 621 *et seq.*

chromosome threads should be more or less generally disposed throughout the nuclear cavity at resting and early prophase stages, and their doubleness should be recognisable from the earliest stages. The nucleolus should show a non-bubbly structure; it should not be surrounded by a clear space, which is due to the shrinkage away from it of other nuclear contents. Spiral thread structures, carrying granules, should be recognisable within metaphase and anaphase chromosomes. The cytoplasm should not shrink.

Pollen mother-cells that cannot be directly exposed to the fixative will generally shrink. In the case of very small anthers and sporangia little can be done to improve the results. Embryo-sacs and deeply immersed megaspore mother-cells of Gymnospermæ and Angiospermæ are also difficult. Every effort should be made to secure direct access of the fixative to the cells that are to be studied.

Fragmentation of long chromosomes, reported in the past by many plant cytologists, is probably an artifact due to the imperfections in the sectioning process, *e.g.* brittle paraffin, dull or saw-edged knife, etc. Chromatin extrusion from resting and early prophase nuclei is probably due in many cases to pressure on the tissue at fixation or cutting or some intermediate stage.

FIXATIVES

1356. Flemming-type fluids are most satisfactory for detailed studies. Fix root-tips, anthers and ovaries twelve to twenty-four hours, smears two to four hours or less. Wash in water, preferably tepid. Bleach in hydrogen peroxide in aqueous or alcoholic solution. Use 1 part of hydrogen peroxide with 4 parts 80 per cent. alcohol, either cold or tepid (stand the jar on thin cardboard on the oven top). GWYNNE-VAUGHAN and BARNES (*The Fungi*, p. 336) use nascent chlorine in 60 per cent. alcohol. Place 1 drop of concentrated hydrochloric acid on a few crystals of potassium chloride and add the alcohol when a green colour indicates the evolution of chlorine. Afterwards wash the slides thoroughly in alcohol.

MCCLUNG (*Anat. Rec.*, xlv, 1918, p. 265) recommends that Flemming fixations should be carried out at 0° C. to keep the tissues unchanged until fixed.

Try medium or strong Flemming solution, Benda with lower acetic acid content, or better, one of the following modifications. It is frequently desirable to use a higher percentage of osmic acid and chromic acid when fixing anthers.

The principal modifications are :—

BONN. 10 per cent. aqueous chromic acid, 0·33 c.c.; 10 per cent. aqueous acetic acid, 3·0 c.c.; 2 per cent. aqueous osmic acid in 2 per cent. chromic acid, 0·62 c.c.; water, 6·27 c.c.

NEWTON and DARLINGTON'S (*J. Genet.*, xxi, 1919, p. 1) formula

is suitable for smears: 1 per cent. chromic acid, 60 c.c.; 2 per cent. osmic acid, 20 c.c.; 10 per cent. acetic acid, 25 c.c.

RANCKEN (*Acta Agralia Fennica*, xxix, 1934, p. 9) uses 30 c.c. of 5 per cent. acetic acid in this mixture for fixing root-tips.

TAYLOR (*Bot. Gaz.*, lxxviii, 1924, p. 236) adds maltose according to the following formula, to assist spreading of the chromosomes and to prevent clumping: 10 per cent. chromic acid, 0·2 c.c.; 10 per cent. acetic acid, 2·0 c.c.; 2 per cent. osmic acid in 2 per cent. chromic acid, 1·5 c.c.; water, 8·3 c.c.; maltose, 0·15 grm.

CATCHESIDE'S (*Ann. Bot.*, xlviii, 1934, p. 601) modification is useful on smears of pollen mother-cells with small chromosomes. 10 per cent. chromic acid, 3 c.c.; 10 per cent. acetic acid, 2 c.c.; 2 per cent. osmic acid, 1·5 c.c.; maltose, 0·2 grm.; water, 10 c.c.

LA COUR'S (*Nature*, cxxiv, 1929, p. 127) two formulæ give chromosomes well spread and constrictions well marked. 2B is 1 per cent. chromic acid, 90, c.c.; potassium bichromate, 1 grm.; sodium sulphate, 0·5 grm.; urea, 1 grm.; 5 per cent. acetic acid, 10 c.c.; 2 per cent. osmic acid, 15 c.c.,; distilled water, 45 c.c.

LA COUR (*J. Roy. Micr. Soc.*, li, 1931, p. 119) gives two further formulæ; 2BE is best for root-tips and smears, 2 BD is useful after Carnoy by Kihara's method. 2BE: 1 per cent. chromic acid, 90 c.c.; potassium bichromate, 1 grm.; saponine, 0·05 grm.; 5 per cent. acetic acid, 10 c.c.; 2 per cent. osmic acid, 15 c.c.; distilled water, 45 c.c. 2 BD.: 1 per cent. chromic acid, 100 c.c.; 1 per cent. potassium bichromate, 100 c.c.; saponine, 0·1 grm.; 5 per cent. acetic acid, 30 c.c.; 2 per cent. osmic acid, 30 c.c.

BĚLAŘ'S modified Flemming-Benda (*Meth. Wiss. Biol.*, i, 1929, p. 638) is satisfactory with smears: 2 per cent. osmic acid, 4 c.c.; 1 per cent. chromic acid, 15 c.c.; acetic acid, 2 to 3 drops.

SMITH (*J. Genet.*, xlix, 1935, p. 119) gives two modifications for pollen mother-cells, S1 for early prophase and S2 for diakinesis and metaphase stages.

	S1	S2
1 per cent. chromic acid .	110 c.c.	75 c.c.
2 per cent. osmic acid . .	35 c.c.	25 c.c.
5 per cent. acetic acid . .	25 c.c.	12·5 c.c.
Potassium bichromate. .	0·5 grm.	1 grm.
Saponine	0·05 grm.	0·05 grm.
Distilled water . . .	50 c.c.	46 c.c.

Various substitutes for osmium tetroxide have been employed.

CARPENTER and NEBEL (*Science*, lxxiv, 1931, p. 154) recommend ruthenium tetroxide. Prepare the stock solution by breaking a 1 grm. ampoule under 100 c.c. of chlorine water. For use, dilute about twenty times with distilled water or a 0·25 to 1 per cent. solution of formic or acetic acid. The correct concentration is that which gives a medium grey colour when a drop is placed on filter paper.

CATCHESIDE (*Genetica*, xvii, 1935, p. 313) uses uranium trioxide in place of osmium tetroxide in the standard formulæ. Material then requires bleaching with hydrogen peroxide (1 part to 4 parts of 80 per cent. alcohol) or with nascent chlorine. Subsequently mordant with 1 per cent. aqueous chromic acid before staining, or use La Cour's gentian violet-iodine-chromic acid technique. See also NEBEL (*Zeits. Zellf. mikr. Anat.*, xvi, 1932, p. 251).

1357. S. NAVASHIN'S (*Mem. Soc. Nat. Kiev.*, 1912, p. 28) **Fixative** is a Flemming formula in which the osmic acid is replaced by an equal volume of 40 per cent. formalin. He used 15 parts of 1 per cent. chromic acid, 4 parts of 40 per cent. formalin and 1 part of glacial acetic acid. In a formula now commonly used the chromic acid is reduced to 10 parts. For an analysis of the fixing action, see LEVITSKY, *Bull. Appl. Bot. Gen. and Plant Breed.*, xxvii, 1931, p. 175.

The principal modifications are: KARPECHENKO (*J. Genet.*, xiv, 1924, p. 387), 1 per cent. chromic acid, 15 parts; glacial acetic acid, 1 part; 16 per cent. formalin, 3 parts; distilled water, 17 parts.

BELLING'S (*The Use of the Microscope*, p. 234):

Solution A: chromic acid crystals, 5 grm.; glacial acetic acid, 50 c.c.; distilled water, 320 c.c.

Solution B1 (for prophase stages): commercial formalin, 200 c.c.; distilled water, 175 c.c.

Solution B2 (for metaphase stages): commercial formalin, 100 c.c.; distilled water, 275 c.c.

For use, take equal parts of solutions A and either B1 or B2, according to the stage of meiosis being fixed; the stage is predetermined by examination of one anther or part of an anther in a drop of iron aceto-carmine. Always use the mixture freshly prepared; it deteriorates rapidly. Fix three hours; a longer period (*e.g.*, twelve hours) is not injurious. Transfer slides to a dish containing solution A only for ten minutes; a longer stay is injurious. Remove anther fragments and *débris*. Rinse in water. Pass slides through 15, 30 and 50 per cent. alcohols (five minutes each), into 70 per cent. alcohol and leave overnight.

WEBER (*Univ. Calif. Pub. Bot.*, xi, 1930, p. 319).

Mix equal parts of solutions A and B.

Solution A: glacial acetic acid, 10 c.c.; chromic acid, 1 grm.; water, 65 c.c.

Solution B: formalin, 40 c.c.; water, 35 c.c.

RANDOLPH (*Stain Tech.*, x, 1935, p. 95) uses equal parts of solutions A and B in:

Solution A: CrO_3 (chromic anhydride), 1 grm.; glacial acetic acid, 7 c.c.; distilled water, 92 c.c.

Solution B: neutral formalin, 30 c.c.; distilled water, 70 c.c.

Fix (root-tips) twelve to twenty-four hours and then transfer

direct to several changes of 70 per cent. alcohol, fifteen minutes in each.

Němec omits acetic acid and uses 25 c.c. of 1 per cent. chromic acid with 2 c.c. of formalin. Use fresh and fix about six hours; then pour off and replace with a fresh portion to act for a further eighteen hours. Reduce the time for delicate structures and organisms. It is erratic in behaviour.

See also: Licent, *C. R. Acad. Sci.*, Paris, clxx, 1920, p. 1518; Pujiula, *J. Broteria Sci. Bot.*, xxi, 1924, p. 90; and *Bull. Inst. Catalana d'Hist. Nat.*, ii, 1925, p. 168; Langlet, *Svensk. Bot. Tidsk.*, xxi, 1928, p. 397; Delaunay, *Mem. Soc. Nat. Kiev*, xxv, 1915, p. 64.

1358. Dehydrating fixatives are suitable for rapid methods (Heitz and McClintock), or used as a pre-treatment in conjunction with an aqueous fixative following Kihara's method.

Yasui (*Cytologia*, v, 1933, p. 140) finds ethyl alcohol, in concentrations from 70 per cent. to absolute, satisfactory for fixing pollen mother-cell smears. It is very suitable for use with Fuelgen's Nuclealfarbung. Of alcoholic combinations, Carnoy is widely used. Farmer and Shove (*Quar. J. Micr. Sci.*, xlviii, 1905, p. 559) have given two modifications, a weaker solution (6 parts absolute alcohol to 1 part glacial acetic acid) and a stronger solution (2 parts absolute alcohol to 1 part glacial acetic acid). Carnoy and Lebrun is also satisfactory. Fixation is rapid. Wash afterwards in 95 per cent. alcohol.

Of other fixatives, Gilson, Helly modification of Zenker, Hermann, Juel and Merkel may be tried if necessary.

Some of Allen's modifications of Bouin (picro-formol-acetic), particularly B–15 and B–3, are useful. Fix ten minutes to a few hours, according to the material. Wash in water and grade up to 70 per cent. alcohol. Hold here a few days, using frequent changes of 70 per cent. alcohol to which a few drops of saturated lithium carbonate solution has been added. Following Bouin fixations, brilliant staining with Heidenhain's hæmatoxylin is obtainable, but gentian violet-iodine is difficult. P.F.A.–3 (B–3 without chromic acid) is very useful since material can be left in it for long periods without danger of over-fixation.

Skovsted (*Ann. Bot.*, xlvii, 1933, p. 227), working on *Gossypium* in the tropics, recommends pre-fixation in Winge's Picro-Carnoy (*Zeits. Zell. Mikr. Anat.*, x, 1930, p. 683), followed by an osmic acid modification of Allen's Bouin. Remove calyx and top of corolla of buds and place five to fifteen minutes in Picro-Carnoy at 37° C.

Picro-Carnoy is:

8 per cent. mercuric chloride in absolute alcohol	1 part
Chloroform	2 parts

A mixture of 100 c.c. 7 per cent. urea in absolute alcohol and 33 c.c. glacial acetic acid . . . . . 3 parts

Transfer to the following fixative, freshly prepared, for twenty to twenty-four hours at 37° C. :

Mix 75 c.c. saturated aqueous picric acid, 5 c.c. glacial acetic acid and 2 grm. urea, heat to 37° C. and add 1·5 grm. of chromic acid. To 3 parts of this, add 1 part each of 2 per cent. osmic acid and 40 per cent. formalin. Wash in 70 per cent. alcohol.

1359. Time at which to Fix. Mitosis is a relatively rapid process and in root-tips shows a diurnal variation in its frequency. Kellicot (*Bull. Torr. Bot. Club*, xxxi, 1904, p. 529) found *Allium* root-tips had a maximum of divisions at 1 p.m. and 11 p.m., and a minimum at 7 a.m. and 3 p.m. Karsten (*Zeits. Bot.*, x, 1918, p. 1) found no rhythm in *Vicia* and *Zea* roots.

See also Friesner, *Amer. J. Bot.*, vii, 1920, p. 380 ; Stalfelt, *K. Svensk. Vet. Akad. Handl.*, lxii, 1921, p. 1.

In general it is unimportant at what time of day root-tips are fixed.

Meiosis is a much more prolonged process. Pachytene lasts several days, but the later stages from diakinesis onwards are gone through fairly rapidly. In the pollen mother-cells of many plants the late prophase and metaphase stages are most frequently found between 9 a.m. and 2 p.m. They cease earlier in warmer weather.

1360. Fixation of Root-tips. Fresh roots are taken from an actively growing plant. Collection is easiest from potted plants when the roots have reached the outside of the ball ; such tips are usually free from soil particles. To obtain root-tips from plants growing in the experimental field it is a good plan to dig a hole close beside the plant, fill it with a rich light compost (free of weed seeds) and keep the plant well watered ; active root-tips may be obtained in two or three weeks. The tips should be removed about 5 to 6 mm. from the apex with a pair of sharp forceps and immediately plunged into a small bottle holding about 10 c.c. of the fixative. When enough tips have been collected, place the bottle under a vacuum pump for a few minutes to exhaust the air and aid penetration. Large roots should be split longitudinally. Air roots with a mucous layer and roots with a suberised root-cap should have slices shaved off their sides with a safety razor blade. Kagawa (*Proc. Imp. Acad. Japan*, iii, 1927, p. 304) finds that if root-tips are narcotised in dilute aqueous solution of chloral hydrate or ether and washed in running water before fixation, the chromosomes appear thicker and their constrictions more clearly defined. Fix in a Flemming or Navashin fluid.

1361. Fixation of pollen mother-cells may be carried out by an aceto-carmine method (*q.v.*), a smear method (*q.v.*), or fixation of

anthers or of whole buds. Anthers are dissected out from buds and plunged immediately into the fixative, preferably of a Flemming type. The Flemming fixatives when used for anthers require a higher percentage of acetic and osmic acids than in the standard formulæ. If the anthers are larger, cut them into small pieces with a sharp scalpel under the fixative. Exhaust all air from the tissues with a vacuum pump.

Kihara (*Bot. Mag. Tokyo*, lxi, 1927, p. 124) finds that cooling of pollen mother-cells before fixing leads to preparations with better-spaced chromosomes. Cool selected buds, put in glass tubes, in running water at 12·5° C. for six hours; or, place the whole plant or shoot in water in an ice chest at 7° to 8° C. for twelve to twenty-four hours. More prolonged treatment (for forty-eight hours) has an injurious effect.

In fixing whole buds, strip off bracts and as much of the perianth as possible, opening up the structure so that the fixative has ready access to the anthers.

Kihara (*Journ. Genet.*, xx, 1928, p. 105) merely removes the bracts and leaves the perianth covering the anthers. The buds are then placed in Carnoy for one to two minutes according to their size and nature; the Carnoy is poured off and is replaced immediately by Bouin, Navashin or a Flemming fixative. If possible the sepals are removed just before washing, to aid infiltration and section cutting. The Carnoy-Flemming method is also given by Tahara (*Bot. Mag. Tokyo*, xxix, 1914, p. 491).

For division of the generative nucleus in the pollen tubes remove them from the style and fix and stain on the slide (O'Mara, *Bot. Gaz.*, xciv, 1933, p. 567).

1362. Ovaries, for megasporogenesis, should have the ovary wall dissected away. Slices may also be cut away from the row of ovules to expose the deeper layers of the nucellus. Cut large ovaries into thin slices. Split those with axile placentation longitudinally and imbed the rows of ovules separately to obtain the correct orientation of each row.

1363. Dehydration and Imbedding. Washing is better done in several changes of tepid tap-water than in running water. After fixation is complete, pour off the fixing fluid and replace by several changes of tap-water. Then stand the material, in tap-water, on a piece of cardboard on top of the paraffin oven, or on a hot plate at about 30° C. Change the water half-hourly. Washing is complete in two to three hours, though flower buds may need four to five hours. Careful dehydration is important. La Cour recommends a series from 10 per cent. by 10 per cent. grades to 80 per cent., 95 per cent. and absolute. Chamberlain suggests a closer grading at the lower end, 2½, 5, 7½, 10, 15, 20, 30 per cent., etc. It is important that the lower grades up to 40 per cent. alcohol should be passed through rapidly. Material may be left in 70

per cent. alcohol, though it is better to add 5 to 10 per cent. of glycerin if it is to be left for a long period. Do not leave longer than twelve hours in absolute alcohol, and in the case of larger buds use two or three changes. Chloroform is preferable to xylol for clearing and infiltration, as it does not harden so much and evaporates more quickly. The time in the oven at a high temperature is thereby shortened. When in pure chloroform, add a small piece of wax and place the phial on the paraffin oven on a piece of cardboard. Leave here three to five days, with the stopper in the phial; add a small piece of wax each day, but never more than will go into solution. Place inside the oven for two hours. Pour into an open dish or watch-glass and allow the chloroform to evaporate off; this usually requires four to five hours. Then imbed.

N-butyl alcohol can be used on root-tips and buds (ZIRKLE, *Science*, lxxi, p. 103; see § 1257) and is advantageous since material can be taken up into wax in a shorter time and the material is not rendered brittle. In LA COUR's (*Journ. Roy. Micr. Soc.*, li, 1931, p. 119) modification material is taken up to pure *n*-butyl alcohol by Zirkle's method and then placed successively for one hour in each of 25, 50, 75 per cent. chloroform in *n*-butyl alcohol, and then into pure chloroform with wax.

Use a harder wax for cutting in the summer (54° to 56° C. m.p. if necessary) and a softer wax for cutting in winter (50° C. m.p. is usually satisfactory). Sections should be cut of sufficient thickness to preclude a high proportion of cut cells; 15 to 20 μ is sufficient for most plants, but 30 μ or thicker is required for those with long chromosomes.

1364. Stains. NEWTON'S **Gentian Violet (or Crystal Violet) Iodine Method** (NEWTON, *Journ. Linn. Soc., Bot.*, lxvii, 1927, p. 346; HUSKINS, *Journ. Genet.*, xviii, 1927, p. 315; NEWTON and DARLINGTON, *Journ. Genet.*, xxi, 1929, p. 1). Bring slides down to water and steep in gentian-violet solution (1 per cent. boiled and filtered) for three to ten minutes according to the age of the stain. Rinse in water and mordant thirty to forty-five seconds in 80 per cent. alcohol containing 1 per cent. iodine and 1 per cent. potassium iodide. Rinse two seconds in 95 per cent. alcohol. Rinse two to five seconds in absolute alcohol. Differentiate in clove oil; clear in three changes of xylol, with at least fifteen minutes in the last change before mounting in xylol-balsam. MANTON (*Ann. Bot.*, xlvi, 1932, p. 509) also mordants with iodine before staining.

For material difficult to stain, and after certain fixatives (Carnoy, Bouin) LA COUR (*Journ. Micr. Soc.*, li, 1931, p. 119), after staining in gentian violet, mordants in iodine solution two minutes, rinses two seconds in absolute alcohol, fifteen seconds in 1 per cent. aqueous solution of chromic acid, five seconds in

absolute alcohol, a further fifteen seconds in 1 per cent. aqueous chromic acid, ten to fifteen seconds in absolute alcohol, differentiates in clove oil and clears in xylol.

After Carnoy fixation, CLAUSEN (*Ann. Bot.*, xliii, 1929, p. 741) mordants slides one to two hours in 1 per cent. aqueous chromic acid and then washes thirty minutes or more in water before staining with gentian violet-iodine or Heidenhain's hæmatoxylin. Alternatively, mordant material fixed in Bouin, Carnoy or Ohlmacher in fresh or used Flemming fixative (ALLEN, *Stain. Tech.*, ii, 1927, p. 62). Or, mordant in 0·5 per cent. aqueous osmic (FOLEY, *Anat. Rec.*, lxiii, 1929, p. 171).

When gentian violet is used as a somatic chromosome stain, JOHANSEN (*Stain. Tech.*, vii, 1932, p. 17) adds 9·5 per cent. of picric acid crystals to the dehydrating alcohols. A much better differentiation completed in clove oil is possible.

SMITH (*Stain. Tech.*, ix, 1934, p. 95) mordants ten to twenty minutes in iodine solution, rinses in water, stains in gentian violet five to twenty minutes and rinses in water. He then rinses in a second iodine solution, then in 95 per cent. alcohol and floods the slide quickly with a saturated solution of picric acid in absolute alcohol, followed by immediate washing with absolute alcohol for a few seconds. Finally, differentiate in clove oil and soak at least ten minutes in xylol. The chromosomes are richly stained by this method and are less transparent than when stained by the ordinary method. Resting nuclei and prophase strands are almost as sharply stained as fully formed chromosomes. The cytoplasm is a very transparent yellow.

PETO (*Journ. Genet*, xxviii, 1933, p. 113) states that the use of distilled water and acid tap-water inhibits staining by the gentian-violet-iodine method. ALVAREZ (*Science*, lxxiv, 1931, p. 633) finds that distilled water in equilibrium with the air absorbs enough CO_2 to lower its *p*H to 5·6 to 5·8. Peto finds that treatment with $\frac{N}{100}$ calcium hydroxide is satisfactory for producing a good stain.

1365. Heidenhain's Hæmatoxylin. This stain is opaque and should be avoided except for prophases of meiosis and for small chromosomes when these are not too numerous. It is unsatisfactory for side views of metaphase and other condensed stages.

For plant material the classical method is as follows: mordant, one to twelve hours in 4 per cent. iron alum; wash five to fifteen minutes in running (or frequently changed) water and stain one to twelve hours in 0·5 per cent. hæmatoxylin. Rinse in water and differentiate in 2 per cent. iron alum, observing the final stages under the microscope. Wash thoroughly fifteen minutes to one hour in water, dehydrate by gradual stages, clear in xylol and

mount in balsam. Use only good iron-alum crystals of pure violet colour for making up the mordant and differentiator.

More recent application of the method has tended towards a shortening of the times as much as possible. Mordant, fifteen to thirty minutes or even eight to ten minutes if the temperature is high; stain for five minutes or more according to the material.

Of rapid methods, COLE's (*Science*, lxiv, 1926, p. 452) is the best. Prepare, as mordant, a solution of 20 c.c. 50 per cent. alcohol, 1 grm. ferric chloride and 2 c.c. glacial acetic acid, and as stock stain 20 c.c. absolute alcohol, 0·2 grm. sodium hydrosulphite, 5 drops distilled water and 1 grm. light brown hæmatoxylin crystals. The stock stain is very powerful and will keep without oxidation for a long time. Use mordant and stain separately or combined. To prepare the stain for use: (1) Add 5 drops of stock solution, followed by 1 drop of ammonium hydroxide, to 5 c.c. of tap-water in a dropping bottle; the stain is ready for use in thirty seconds and retains its potency for about four hours. Or, (2) use 95 per cent. alcohol instead of tap-water; the stain is ready for use after twenty minutes and has a longer life than the aqueous solution. Or, (3) add 5 drops of stock solution to 10 drops of tap-water and add 1 drop of ammonium hydroxide; after thirty seconds add 5 c.c. 95 per cent. alcohol and the stain is ready for immediate use and lasts longer than (1).

Method. Flood the slide with mordant for five minutes. Rinse a few seconds in tap-water and flood with stain. Staining requires ten minutes or less. Differentiate in 0·1 to 0·4 per cent. hydrochloric acid and when this is complete blue the slide in a jar of water containing 1 or 2 drops of ammonium hydroxide. Counterstain in erythrosin, if desired. Dehydrate, clear and mount. The fresh stain gives blue nuclei; that an hour or more old gives blue-black or black nuclei. To secure black nuclear stains use solutions several hours old, or add 2 to 3 drops of ammonium hydroxide in the preparation of the stain for use, or flood slides with fresh or old stain and add 1 drop of ammonium hydroxide.

See also FAURE (*C. R. Soc. Biol.*, xc, 1924, p. 87); KAUFMANN (*Stain. Tech.*, ii, 1927, p. 88); KORNHAUSER (*Stain Tech.*, i, 1926, p. 78); MAHESHWARI (*Journ. Ind. Bot. Soc.*, xii, 1933, p. 129).

TUAN (*Stain Tech.*, v, 1930, p. 135) recommends the use of a saturated aqueous solution of picric acid to differentiate material stained in Heidenhain's and Delafield's hæmatoxylin; follow by washing thirty minutes in water. A more transparent stain is produced, no brownish or muddy colouration is obtained, and since picric acid is milder in its destaining properties a more controlled action is possible.

1366. MAYER's **Hæmalum.** Grind up 0·5 grm. hæmatein in a glass mortar with 10 c.c. of 95 per cent. alcohol and add 500 c.c. of 5 per cent. aqueous solution of potassium alum (see KORNHAUSER,

Stain Tech., v, 1930, p. 13). Stain, from water, for five minutes. Wash in running water, or several changes of tap-water, dehydrate, clear and mount.

SASS's (*Stain Tech.*, iv, 1929, p. 127) **Modification of Mayer's Hæmalum.** Dissolve 50 grm. ammonium alum in 1 litre of boiling water. Remove from hot plate and add 1 grm. hæmatoxylin and 1 grm. sodium iodate ($NaIO_3$), cool and filter. The stain keeps several months, but is best used when fresh. Filter whenever a "metallic" scum appears. Transfer to stain from water, wash in distilled water, then in tap-water (or 0·001 per cent. sodium carbonate), and again in distilled water; dehydrate, clear and mount. The stain is primarily an histological one. By adding to the stain alum to saturation and 2 to 5 per cent. of acetic acid, nuclear selectivity is increased. Chromosomes of *Lilium* ovary in meiosis are stained in one hour and require no differentiation.

1367. Feulgen's Nucleal-färbung may be used. See FUELGEN (*Handbuch Biol. Arbeitsmeth.*, ccxiii, 1926, p. 1055), for detailed discussion of methods.

GURNEY (*Australian J. Exp. Biol. and Med. Sci.*, xi, 1933, p. 157) obtains brilliant staining of wheat anthers cut at 10 μ. Fix in Carnoy, in saturated mercuric chloride ($HgCl_2$) in 2 per cent. acetic acid or in a mixture of equal parts of chromic acid and formalin. Fix sections to the slide and hydrolyse with N hydrochloric acid for ten minutes at 60° C. Cool the acid by immersing the vessel in running water and then transfer the slides to cold acid; this prevents sections floating off the slide. Stain for two hours; all chromatic material takes a deep purple colour seen only in daylight. Use a Wratten green B filter with artificial light.

Fuelgen's method can be applied to unfixed pollen mother-cell smears; the N hydrochloric acid solution then appears to serve as a coagulator of the cell contents.

1368. Safranin. Though red stains are usually best avoided, the following method gives useful results. TUAN (*Stain Tech.*, v, 1930, p. 103) stains sections and smears in safranin and dehydrates in a series of alcohol solutions containing 1·5 per cent. picric acid and constantly decreasing percentages of water. Differentiation is chiefly effected in 83 per cent. alcohol containing 1·5 per cent. picric acid and completed in the final dehydrating and clearing. Counterstain in clove oil if desired.

See also GEITLER, *Züchter*, i, 1929, p. 243.

THALER (*Mikrokosmos* (Stuttgart), xx, 1927, p. 203) describes the technique for the following nuclear stains: gallamin blue, coelest blue, gallocyanin, coerulein A and gallein.

Stain combinations are rarely used in modern chromosome studies. For some possible methods see Cytological Stains.

1369. Belling's **Iron Aceto-carmine Method** (*Amer. Nat.*, lv, 1921, p. 573). Belling describes three procedures: (1) Heat 45 per cent. glacial acetic acid to boiling with excess powdered carmine, cool and filter; 1 grm. of carmine to 100 to 200 c.c. of 45 per cent. acetic acid is sufficient. Do not boil more than half a minute. Tease out anthers in a drop of the aceto-carmine solution with steel blades or needles until the colour changes toward a bluish-red. Remove anther *débris*, cover with a large thin coverslip, using a minimum of liquid. Seal the edges with vaseline. The slide may improve in a day or two if no excess of iron is present. (2) Add a trace of ferric hydroxide dissolved in 45 per cent. acetic acid until the liquid turns bluish-red, but there is no visible precipitate. Add an equal amount of ordinary aceto-carmine. Tease anthers with nickel instruments. If the stain is too dark, add more aceto-carmine. The liquid may be diluted with 45 per cent. acetic acid, never with water. Where the stain is too heavy, McClintock (*Genetics*, xiv, 1929, p. 180) extracts with dilute acetic acid, warming the whole slide gently to hasten the action. (3) Anthers at the right stages are put into a mixture of 1 part of glacial acetic acid to 9 parts absolute alcohol, to which sufficient ferric hydroxide in 45 per cent. acetic acid has been added to colour the liquid brown. After some days or even weeks, the anthers are teased in ordinary aceto-carmine, avoiding the use of steel instruments.

Belling (*Amer. Nat.*, lvii, 1923, p. 92) found advantages in using a water immersed aplanatic condenser with a water immersion objective. Approximately monochromatic yellow-green light, obtained with a colour filter (Wratten 57a or 58, or green B) is useful.

Belling later (*Biol. Bull. Marine Biol. Lab.*, l, 1926, p. 160) recommends a solution of dammar in xylol or melted soft paraffin wax as a seal for the edge. Chloral hydrate, followed by mounting in glycerin, may be used for clearing pollen grains.

The best seal is prepared by heating together equal parts by weight of paraffin and gum mastic. Apply with a hot wire.

The pollen mother-cells fixed and stained in iron aceto-carmine pass through three stages of hardening (Belling, *Brit. Jour. Exp. Biol.*, iii, 1926, p. 145). At first the cytoplasm is more or less liquid, then it becomes a more or less stiff jelly and finally (after some weeks) more and more brittle; the chromosomes are always harder than the cytoplasm. If the cells in the second stage (reached in one to two days) are subjected to gradual pressure, the cell contents may be squeezed flat within or without the cell-wall. Slides keep better stored in the dark.

Iron aceto-carmine is satisfactory with most material; notable exceptions are *Œnothera* and *Rhododendron*.

McClintock (*Proc. Nat. Acad. Sci.*, xvi, 1930, p. 791) studying

the mid-prophase stages (pachytene) in pollen mother-cells of *Zea Mays*, recommends that the slides be gently heated after the coverslip has been applied. The pollen mother-cells flatten, the nuclear membrane disappears and the long thread-like para-synapsed chromosomes are mostly spread out in a horizontal plane.

SAX and SAX (*J. Arnold Arboretum*, xiv, 1933, p. 356) dissect out entire endosperms from female cones of Coniferales and fix them in 70 parts absolute alcohol mixed with 30 parts acetic acid. Fix several hours and store in 80 per cent. alcohol. They make aceto-carmine smears from the fixed material. This haploid tissue is very useful for study.

HUSKINS (*Journ. Genet.* xviii, 1927, p. 315) fixed material in Carnoy, stored it in 70 per cent. alcohol and later prepared slides by Belling's second method.

By smearing a single anther, immediately flooding with Belling's aceto-carmine and heating over a spirit flame for a second, the slide is ready for immediate examination to determine the stage of meiosis. Thereafter the rest of the anthers can be rejected or used for further treatment by this or any other method. The worker is thereby saved endless labour, since only material at the correct (or approximately correct) stage is subjected to lengthy techniques.

See also BELLING, "The Use of the Microscope; BELLING, *Journ. Roy. Micr. Soc.*, 1925, p. 445; DE MEYERE, *Zool. Anzeig.*, lxxxviii, 1930, p. 209.

1370. Permanent Iron-Aceto-carmine Preparations in Canada Balsam. First designed by BELLING (*Biol. Bull. Marine Biol. Lab.*, l, 1926, p. 160). See also LONGLEY (*J. Agr. Res.*, xxxv, 1927, p. 769).

McCLINTOCK (*Genetics*, xiv, 1929, p. 180) dried smears slowly and mounted them in balsam. She has also described (*Stain Tech.*, iv, 1929, p. 53) a highly satisfactory method of making permanent aceto-carmine smears. Anthers are collected and put into a mixture of 1 part acetic acid to 3 parts absolute alcohol. Fix twelve to twenty-four hours. The contents of an anther are squeezed out on a slide into a drop of Belling's iron aceto-carmine solution (Belling's second procedure above) and a cover-glass is placed over the drop. Care should be taken to remove all anther walls and other flower parts. Then heat the slide over a spirit flame for a second, repeating four to five times. Next, place the slide in a Petri dish filled with 10 per cent. acetic acid. When the cover-glass has risen away from the slide remove it and place both it and the slide in a Coplin jar containing equal parts of alcohol and acetic acid. Some pollen mother-cells stick to the slide and some to the cover. Run both coverslip and slide through the following solutions: 1 part acetic acid to 3 parts of absolute alcohol, 1 part acetic acid to 9 parts absolute alcohol, absolute

alcohol, equal parts absolute alcohol and xylol and finally into xylol. Recombine coverslip and slide in xylol balsam directly from the xylol.

Buck (*Science*, lxxxi, 1935, p. 75) makes aceto-carmine preparations permanent by placing the slides face down for five to thirty minutes in a mixture of equal parts xylol, absolute alcohol and glacial acetic acid. Remove coverslip and rinse in the same solution for five minutes; drain and wipe clean. Pass through two changes of xylol, absolute alcohol (1 : 1), five to ten minutes each and then xylol ten to fifteen minutes.

Metz and Gay soak off the cover in equal parts of 95 per cent. alcohol, clove oil and glacial acetic acid; wash in two changes of 95 per cent. alcohol, up to half an hour; absolute alcohol, five minutes; clove oil, ten minutes; xylol, five minutes.

Steere (*Stain Tech.*, vi, 1931, p. 107) has described a rapid method in which anthers from a selected bud are smeared and fixed and stained by immediate immersion, face downward (*cf.* Smear Technique) in a Petri dish of hot (steaming) aceto-carmine for 1 to ten minutes. Then rapidly transfer them successively through the following mixtures: 2 parts of 99 per cent. (glacial) acetic acid with 1 part of absolute alcohol, 1 part acetic acid with 2 parts alcohol and 1 part acetic acid with 9 parts of absolute alcohol into absolute alcohol, where the slides are dehydrated for one to two minutes. Clear two to three minutes in equal parts of xylol and absolute alcohol, then xylol and mount in balsam. The whole process requires five to fifteen minutes and the slides are suitable for chromosome counts, etc.

See also Sax, *Stain Tech.*, vi, 1931, p. 117.

1371. Heitz's Koch Methode (*Zeits. Bot.*, xviii, 1926, p. 625) is a method for rapid counting of chromosomes. Fix anthers or ovaries in hot Carnoy (Farmer's formula: 1 part of acetic acid to 2 parts of absolute alcohol) followed by aceto-carmine (45 per cent. acetic acid boiled with carmine, cooled and filtered). Subsequently the material is teased apart with needles and left some hours for the stain contrast between chromosomes and cytoplasm to intensify. Slides may be dehydrated or dried after staining and mounted in balsam.

Sinôto (*Cytologia*, i, 1929, p. 109) fixes anthers and root-tips in aceto-alcohol (1 part acetic acid to 2 or 3 parts absolute alcohol) and heats them in a tube till the solution begins to boil. The fluid is poured off and replaced with Schneider's aceto-carmine and boiled again. Portions of material are mounted on a slide and covered and squeezed with a pincett to crush the tissues. When overstained the material is destained in 45 per cent. acetic acid to the desired degree.

De Meijere (*Zeits. wiss. Mik.*, xlvi, 1929, p. 189) treats pollen mother-cells after Heitz's method with Carnoy, places

them in aceto-carmine under a cover-glass and heats them to boiling point. The aceto-carmine is then replaced with glacial acetic acid, by an irrigation method with the aid of filter-paper, and the acetic acid with Venetian turpentine.

The chromosomes in young, vigorous root-tips can be examined by the method of WHITAKER (*Stain Tech.*, ix, 1934, p. 107). Tips are fixed in aceto-alcohol (1 part glacial acetic acid, 2 parts absolute alcohol) for twenty-four to forty-eight hours. The material can be stored indefinitely by transference to 80 per cent. alcohol. To prepare the smears, place the root-tips on a slide and slice them as thinly as possible with a sharp razor blade. Then smear the slices on the slide, immediately flood with aceto-carmine and add a coverslip. Using absorbent paper and exerting considerable pressure, the excess aceto-carmine can be removed and the material flattened at the same time. Finally, warm the slide to a point slightly below boiling; this aids contrast. Sealed with gum mastic and paraffin, such preparations keep well for five to ten days.

WARMKE's **Permanent Root-tip Smear Method** (*Stain Tech.*, x, 1935, p. 101). Fix twelve or more hours in 1 part glacial acetic acid and 3 parts absolute alcohol. Then place for five to ten minutes in a solution of 1 part 95 per cent. alcohol and 1 part concentrated HCl to dissolve the pectic substances of the middle lamella. Harden again with Carnoy for five or more minutes. Place a small piece of the root-tip (0·5 mm.) on a slide in a small drop of iron aceto-carmine. Press material with scalpel tip and cover. Press gently with flat blunt end of pencil and then heat three to four times in spirit flame; do not boil. Seal edge of preparation. Preserve good slides by McClintock's method. Pollen grains also satisfactory this way; the heavy exine is split by the HCl, leaving the naked protoplast adhering to the slide.

Probably other carmine stains could be used successfully following aceto-alcohol fixation. FYG (*Zeits. wiss. Mikr.*, xlv, 1928, p. 442) gives formulæ for the preparation of carmine stains using chrome alum, aluminium sulphate, copper alum, sodium bicarbonate, ferric oxide and ammonium sulphate as mordants. The first three give nuclei a violet or blue-black colour. See also SCHMELZER (*Zeits. wiss. Mik.*, li, 1934, p. 66).

BARRETT (*Stain Tech.*, vii, 1932, p. 63) uses Heidenhain's hæmatoxylin according to the following formula, for studying chromosomes in temporary smears of pollen mother-cells: 1 part 0·5 per cent. hæmatoxylin and 4 per cent. iron alum (equal parts of each), 1 part of 95 per cent. alcohol and 2 parts glacial acetic acid.

1372. Permanent smears of plant material were first made by TAYLOR (*Bot. Gaz.*, lxxviii, 1924, p. 236) who used Flemming (Taylor's modification) followed by Heidenhain's hæmatoxylin.

Crush anthers on clean slides with a clean flat-honed scalpel, and spread their contents over the centre of the slide with quick strokes. Immediately invert the slide in the fixing fluid, bringing it down in a horizontal position, so that the whole smear face is wet simultaneously. The time from first crushing to the fixing must not exceed three to five seconds. If the slide is brought down obliquely, much of the material washes off. Fix in a Petri dish with a thin glass rod in the bottom, sufficient liquid being used just to cover the rod. Square porcelain dishes ($3\frac{1}{4} \times 3\frac{1}{4}$ in.), with two small ridges near opposite ends are better. Fix not less than fifteen minutes, wash in water and remove the larger pieces of anther walls and other *débris.* Bleach in dilute aqueous hydrogen peroxide, rinse and place four to twelve hours in 2 per cent. iron ammonia alum. Wash fifteen minutes or more in running water, stain four to twelve hours in 0·5 per cent. aqueous hæmatoxylin, followed by a rinse in water and differentiation in iron-alum solution under the microscope. Finally, wash for one hour. Dehydrate through alcohols by 10 per cent. stages, two to three minutes in each. The chief difficulty is to get a brilliant stain, some slides being distinctly muddy.

By KAUFMANN's (*Stain Tech.*, ii, 1927, p. 88) method fewer muddy slides are obtained. Mordant forty-five minutes to one hour in 2 per cent. alum. Wash in running water ten to fifteen minutes. Stain twenty to thirty minutes in 0·25 to 0·5 per cent. hæmatoxylin; as soon as the slides are deep purplish-black transfer to water. Differentiation in 0·5 per cent. ammonio-ferric alum can then more often be completed prior to the appearance of a muddy colour than is possible after prolonged staining. Kaufmann found no advantage in allowing the hæmatoxylin to ripen. He also fixed smears in a picric acid-acetic acid-formaldehyde combination and found organic adjuvants such as lactose, maltose and urea in 1·5 per cent. concentration improved penetration.

NEWTON (*J. Linn. Soc.* (*Bot.*), lxvii, 1927, p. 339) and DARLINGTON (*J. Genet.*, xvi, 1926, p. 237) used gentian violet-iodine as a stain, following fixation in Flemming.

SAX (*Stain Tech.*, vi, 1931, p. 117) fixes smears in Navashin's or modified Flemming solution for one to two hours. Wash in 10 to 30 per cent. alcohol for fifteen to thirty seconds and stain in 1 per cent. aqueous crystal violet one to five minutes. Rinse in water and pass through 30 and 50 per cent. alcohols, fifteen to twenty seconds in each. Transfer to 80 per cent. alcohol (containing 1 per cent. iodine and 1 per cent. potassium iodide) for thirty seconds. Destain with absolute alcohol, followed by clove oil, xylol and balsam.

Observation of preparations stained with gentian violet (or crystal violet) is facilitated by using a green screen (*e.g.*

Wratten B) when the chromosomes appear nearly black. With a yellow screen (Wratten K3) they appear deep red.

Mann (*Science*, lx, 1924, p. 548) has a method of making permanent smears of pollen mother-cells, in which the contents of the anther are distributed with a scalpel over a film of albumen smeared on a slide.

Webber (*Univ. Calif. Pub. Bot.*, xiv, 1929, p. 345) uses a much shortened schedule in which smears are fixed in acetic alcohol and stained with alcoholic stains. All mordanting, staining, washing, etc., is done in 60 per cent. or higher alcohols. Slides stained with Delafield's hæmatoxylin are prepared in one and a half hours, those with iron brazilin in four hours. Useful for chromosome counts and morphology, cytokinesis, tetrad cells, etc., but not for the finer details of prophase. The methods of Mann (*Science*, xxxvi, 1912, p. 151) and Pickett (*Science*, xxxvi, 1912, p. 479) for preparing slides of unbroken pollen mother-cells have now little more than an historical interest.

1373. Belling's **Iron-Brazilin Method** (*Univ. Calif. Pub. Botany*, xiv, 1928, p. 293; *The Use of the Microscope*, p. 243). Smears are made using a scalpel or a second slide held crosswise over the first. Make one rapid slanting or curving sweep with the scalpel or second slide and immediately invert the slide or slides on the fixative. Fix in Belling's modified Navashin, grade into 70 per cent. alcohol and leave overnight. Fixation is rapid except where sap from the anther wall or connective reaches the pollen mother-cells. Mordant twenty-four hours in 1 per cent. ferric ammonia alum in 70 per cent. alcohol. Wash briefly in 70 per cent. alcohol and soak fifteen minutes to three hours in 70 per cent. alcohol; the shorter the time the more deeply the metaphase and cytoplasm stain. Stain two to twenty-four hours in a solution of 0·5 grm. brazilin in 100 c.c. of 70 per cent. alcohol. When using freshly made brazilin solutions, add 1 to 2 drops of 1 per cent. iron-alum solution per 50 c.c. Wash briefly in 70 per cent. alcohol, and differentiate in iron alum in 70 per cent. alcohol for one minute to three hours or more. Pachytene and chromomeres need only slight differentiation as only two to three hours' staining is necessary for them. Stain metaphases for a longer time; they then need longer differentiation, of one or more hours, especially if the chromosomes are small. Next wash slides in 70 per cent. alcohol and transfer to 95 per cent. alcohol. Pass successively through absolute alcohol, equal parts of absolute and thin cedar oil, equal parts of xylol and thin cedar oil into xylol. Mount in immersion cedar oil. Chromomeres and chromosomes should be brown to black, cytoplasm pink or colourless and cell-walls unstained. Use green Wratten filters to heighten the contrast, viz., 57A or 64 (blue-green) or a combination of 56 and 64. The brazilin can be replaced by hæmatoxylin dissolved to

0·5 per cent. in 70 per cent. alcohol; a longer differentiation is needed and the cytoplasm is not so clear.

See also CAPINPIN, *Science*, lxxii, 1930, p. 370.

1374. BACKMAN'S **Combined Fixing and Staining Method** (*Stain. Tech.*, x, 1935, p. 83). Substitute anthraquinone for picric acid in Bouin formula and add alizarin red S :—

Anthraquinone, sat. soln.	75 c.c.
Formaldehyde	25 c.c.
Acetic acid	5 c.c.
Alizarin red S	0·125 grm.
Saponine	0·150 grm.

Immediately before use add 8 to 12 drops of normal solution of metallic salt (*e.g.* aluminium chloride, copper nitrate, ferric chloride). Suitable concentration of salt solution found by adding as many drops to the fixative as it will hold without precipitation of hydroxide during maximum fixing time (three to four hours).

Smear and fix three to five minutes; wash in tap-water two to five minutes; dehydrate to 95 per cent. alcohol. Differentiate by flooding with 0·5 per cent. H_2SO_4 in 95 per cent. alcohol saturated with picric acid; this requires only a few seconds. Wash in 95 per cent. alcohol. If slide to be used at once, intensify stain by washing half to one minute in 95 per cent. alcohol containing 4 per cent. by volume of cymene. Wash in absolute alcohol, clear in xylol and mount. Backman gives an alternative method of differentiating and also a schedule for root-tips.

1375. Internal (Spiral) Structure of Chromosomes at metaphase and anaphase is frequently visible in well-fixed smears of pollen mother-cells from plants with large chromosomes. Fixation with 2BD (La Cour) and S2 (Smith) is particularly good. Spirals are seen particularly well in pollen mother-cells that have been slightly pressed prior to fixation. SAKAMURA (*Bot. Mag.*, Tokyo, lxi, 1927, p. 59) fixes in hot water.

KUWADA and SAKAMURA (*Cytologia*, v, 1934, p. 244) squeeze pollen mother-cells of *Tradescantia* out into a drop of 3 per cent. cane sugar on a coverslip. The coverslip is exposed for a few seconds, material downwards, on a cell containing a small piece of cotton-wool soaked in ammonia and then fixed and stained with iron aceto-carmine. The spirals are spread out, or even unravelled by prolonged treatment. LA COUR (*Stain Tech.*, x, 1935, p. 57) makes smears on slides and carefully wets them by putting them face downwards horizontally on to 3 per cent. cane-sugar solution. The slides must also be removed horizontally or much material washes off. The slides are then exposed to ammonia vapour or the vapour of acetic acid or of mineral acids (nitric or hydrochloric) for a few seconds. He then fixes the smears in a Flemming fluid (medium Flemming slightly better

than Navashin or 2BE) and stains by the gentian violet-iodine method. Permanent smears showing spiral structure are thus obtainable.

Kuwada and Sakamura (*Protoplasma*, i, 1926, p. 239) observed spirals in living pollen mother-cells, injected with carbon dioxide by passing the gas over the material. The *p*H markedly affects the visibility of the spirals. They obtained their best results using a 0·025 per cent. solution of neutral violet extra in a solution of *p*H 4·38, making observations on the peripheral cells of the groups. See also Sakamura (*Protoplasma*, i, 1926, p. 537).

Nebel (*Zeits. Zellf. mikr. Anat.*, xvi, 1932, p. 251) finds that slight desiccation prior to fixation leads to deeply stained spirals. With more prolonged treatment the spirals stain less deeply and with still longer treatment they disappear. Other pre-treatment he recommends are 12 per cent. alcohol accompanied by a temperature increase (Sharp, *Bot. Gaz.*, lxxxviii, 1929, p. 349), 95 per cent. alcohol followed by water, brief treatment with Carnoy before Flemming fixation and $\frac{N}{2}$ Ringer for twenty seconds. He also recommends fixation in a mixture of equal parts each of 1 per cent. uranyl acetate, saturated aqueous mercuric chloride and 50 per cent. formic acid, with the addition of 2 parts of 2·5 per cent. osmic acid. Rinse off the fixative and mount in Linder's medium and seal. Linder's medium is glycerin 40 parts, lactic acid 20 parts, phenol 20 parts, water 20 parts; add a small amount of carmine.

Cohen (*Stain Tech.*, ix, 1934, p. 101) states that fixation with 4 per cent. phosphoric acid in 4 per cent. formaldehyde, followed by the crystal violet-iodine stain, shows details of the internal structure of chromosomes.

Pre-treatment with various inorganic salts, *e.g.* uranium nitrate or potassium nitrate, also appears to be effective.

Preservation of spirals in somatic chromosomes is more difficult, since the material cannot be well fixed by any of the ordinary methods. Try fixation in aqueous Flemming fluids by finely chopping root-tips under the fixative.

Nebel's method (*Cytologia*, v, 1934, p. 1) appears to be the most effective. Place root-tips in 0·1 per cent. solution of commercial ammonia for five minutes. Transfer to a 0·01 molar solution of thorium nitrate and finally fix twenty-four hours in a mixture of 10 parts of 2·5 per cent. osmic acid in 1 per cent. platinic chloride and 3 parts of 1 per cent. aqueous chromic acid.

See also Strugger, *Planta, Arch. Wiss. Bot.*, xviii, 1932, p. 561 for use of pre-treatments; Taylor, *Proc. Internat. Congr. Plant Sci.*, Ithaca, N.Y., 1, 1926, p. 265.

CHAPTER LII

GENERAL TECHNIQUES FOR CLASSES OF PLANTS

ALGÆ

See also Protozoa §§ 1108–1165.

1376. Collection of fresh-water forms should be made into small wide-mouthed tubes or bottles. Three-quarters fill the tube with water and put only a little of the alga in the tube.

WETTSTEIN (*Oesterr. Bot. Zeits.*, lxx, 1921, p. 23) collects into a culture medium. He describes two, one of mineral nutrients alone, the other having a peat decoction in addition. Agar, not over 1 per cent., is added so that the medium does not solidify. Take the medium into the field in bottles and place in it the material collected. Later pour the jelly into plates. The algæ develop into colonies, which can be transferred to other plates. It is often difficult to separate them from bacteria, but for most purposes this is unnecessary.

For methods of collection of plankton, see WEST and WEST, *Proc. Roy. Soc. B.*, lxxxi, 1909, p. 165; SUTHERLAND, *Journ. Ecol.*, i, 1913, p. 166; BACHMANN, *Biol. Centrbl.*, xx, 1900, p. 386; LOHMANN, *Int. Rev. Hydrobiol. u. Hydrogr.*, iv, 1911, p. 1; SCOURFIELD, *J. Queckett Micr. Club*, xi, 1912, p. 243.

1377. Culture. Whenever possible algæ should be kept alive and examined in the living state.

KUFFERATH (*Rev. Algol.*, iv, 1929, p. 127) has a general treatise on the culture of algæ, citing and describing the methods of various authors, including formulæ and isolation technique.

KLEBS (*Die Bedingungen der Fortpflanzung bei einigen Algen u. Pilzen.*, Jena, 1896, p. 8) used Knop's nutrient solution (4 parts calcium nitrate and 1 part each of magnesium sulphate, potassium nitrate and potassium phosphate, used in a dilution of 0·2 to 0·5 per cent.) for fresh-water forms. BRISTOL (*Ann. Bot.*, xxxiv, 1920, p. 35) used 1 grm. potassium dihydrogen phosphate, 1 grm. sodium nitrate, 0·3 grm. magnesium sulphate, 0·1 grm. calcium chloride, 0·1 grm. sodium chloride, a trace of iron chloride, 1,000 c.c. distilled water. Generally a suitable formula must be devised for each alga by a trial.

See also: CUNNINGHAM, *J. Elisha Mitchell Sci. Soc.*, xxxvi., 1921, p. 123, and xxxix., 1923, p. 10 (diatoms); NIEUWLAND, *Midland Naturalist*, i., 1909, p. 85; KUFFERATH, *Ann. Biol. Lacustre*, ix., 1919, p. 1; OEHLER, *Arch. Protistenk*, xl., 1919, p. 16, and xlix., 1924,

p. 287 (flagellates); SKINNER, *Plant Physiol.*, vii., 1932, p. 533 (soil algæ).

Pure cultures are frequently essential, especially for the simple unicellular and colonial forms. Numerous methods have been devised. Due regard must be paid to the sterilisation of all vessels, media, etc., but it is usually difficult to obtain such cultures free from admixtures of bacteria.

See BEIJERINCK, *Bot. Zeit.* xlviii., 1890, p. 725; CHODAT and GRINTZESCO, *C. R. Congr. int. Bot.*, Paris, 1900, p. 157; GRINTZESCO, *Bull. Herb. Boissier*, sér. 2, v., 1902, p. 225; KLEBS, p. 184 (above); RICHTER, *Ber. Deutsch. bot. Ges.* xxi., 1903, p. 493 (diatoms); WETTSTEIN (above).

Reference should be made to the work of Klebs and others for methods of inducing the reproductive phases.

1378. Fixation. Most are adapted to methods applicable to filamentous specimens. Imbedding should be by very gradual stages. Mats of filaments, crusts of filamentous and unicellular types on sticks and stones, and epiphytes and endophytes should be fixed and stained undisturbed. Tease, scrape from the substratum and dissociate as necessary just before mounting.

Shrinkage of the protoplast in filamentous forms is most troublesome. To eliminate, vary the amounts of the various constituents of the fixative, increasing the acetic acid until shrinkage is overcome. Handle unicellular and other small forms by sedimentation methods. Or, place a drop of suspension on an albumened slide, fix with the vapour of osmic acid for a few seconds to one minute. Dry the drop and stain. If the specimens are rare, pick them out with a fine pipette. CHAMBERLAIN (1932, p. 228) handles small amounts of planktonic forms rolled up in a tube made of a strip of epidermis from an inner scale of an onion; the ends of the tube are tied. Cut off the ends when ready to mount. Material can be imbedded and sectioned in this way. See also CONGER, *J. Roy. Micr. Soc.*, 1925, p. 48.

BOLD (*Bull. Torrey. Bot. Club*, lx, 1933, p. 241) fixes *Protosiphon* (coenocytic), grown on agar, *in situ*, by placing drops of fixative on the plants with a pipette. Then pour melted agar, near the point of gelation over the fixed plants. When the agar has set, cut out blocks containing the alga and imbed in paraffin.

Handle massive forms (Siphonocladiales, etc.) like soft tissues of higher plants. Decalcify if calcareous.

Fix in 4 per cent. formalin, chromo-acetic, Bouin or formalin-acetic-alcohol for habit and grosser cell structure. G. H. C. (*Turtox News*, iii, p. 45) recommends Rawlin's formol-acetic-alcohol (see Rawlins, p. 14), in which *Cladophora* alone sometimes shows plasmolysis. EVAN'S, KEEFE'S or WOOD'S (*Science*, lxx, 1929, p. 637) fluids will preserve green algæ in their natural colour. WEST (*ex* NIEUWLAND, *Bot. Gaz.*, xlvii, 1909, p. 237) fixes pre-

serves and mounts in a 2 per cent. solution of potassium acetate, just made blue with a small amount of copper acetate; for mounting, Nieuwland adds an equal volume of 10 per cent. glycerin and allows it to concentrate.

Flemming type fluids are best for details; use the weaker formulæ, especially on filamentous forms.

Sublimate fixatives give good results with algæ, and are frequently best used hot. Try a saturated solution of corrosive sublimate in 70 per cent. alcohol (Fergusson, *Ann. Bot.*, xlvi, 1932, p. 703). Carter (*Ann. Bot.*, xxxiii, 1919, p. 213) fixes desmids in a mixture of 3 grm. corrosive sublimate, 3 c.c. glacial acetic acid and 100 c.c. 50 per cent. alcohol; use hot and replace at once with several changes of 50 per cent. alcohol. For Cladophoraceæ, reduce the acetic acid to 1 c.c. (Carter, *Ann. Bot.*, xxxiii, 1919, p. 467).

Many Myxophyceæ do not fix well. *Oscillatoria* and hormogonia of other genera can be induced to creep on to a slide; then fix *in situ*. Large fleshy Phæophyceæ and Rhodophyceæ should be cut into small pieces for accurate fixation.

Fix marine forms in solutions made up in sea-water. For general preservation use 4 c.c. formalin (40 per cent. formaldehyde) in 96 c.c. sea-water, or for more critical work 25 c.c. 1 per cent. chromic acid in sea-water, 100 c.c. 1 per cent. glacial acetic acid in sea-water, 65 c.c. sea-water. Higgins (*Ann. Bot.*, xlv, 1931, p. 345) doubles the volume of acetic acid used in the case of some forms (*e.g.*, *Stypocaulon*) and reduces the sea-water by 10 c.c. Wash in sea-water and transfer to fresh water (see dehydration). Preserve in 50 c.c. alcohol, 4 per cent. formalin, Keefe, or glycerin.

Alsterberg (*Bot. Notiser*, 1927, p. 71) finds bromocyanin an excellent preservative of filamentous algæ.

Diatoms encrusting material can be removed with hydrofluoric acid.

See also von Wellheim, *Jahrb. Wiss. Bot.*, xxvi, 1894, p. 674. Lemmermann, *Kryptogamenflora Mark Brand*, iii, 1910, p. 12; Smith, *Plant World*, xvi, 1913, p. 219; Tiffany, *Trans. Amer. Micr. Soc.*, xlv, 1926, p. 69; Lucas, *Science*, lxx, 1929, p. 482; Lebour, "The Dinoflagellates of Northern Seas," Marine Biol. Assoc. U.K., Plymouth, 1925, 250 pp.; Chamberlain, *Publ. Puget Sound Biol. Stat.*, V, 1928, p. 319 (microtechnique for marine algæ).

1379. For preserving Myxophyceæ and Rhodophyceæ in their natural colours, Kirchner (*Die mikr. Pflanzenwelt des Süsswassers. Braunschweig*, 1885) mounts in dilute glycerin, to which sufficient chrome alum (chromium-potassium sulphate) is added to give the fluid a clear bluish colour.

1380. Staining. Most useful are Heidenhain's hæmatoxylin,

Mayer's hæmalum, safranin and phloxine (Magdala red); counterstain (if necessary) with erythrosin, orange G., anilin blue, cyanin or light green. Newton's gentian violet-iodine gives excellent results on sections and filamentous types.

After staining, dehydrate whole material in glycerine and mount in Venetian turpentine or Canada balsam. G.H.C. (*Turtox News*, iii, p. 45) washes out the glycerin with absolute methyl alcohol, to which a very little light green is added.

Geitler (*Österr. Bot. Zeits.*, lxxi, 1922, p. 116) advocates fixing living algæ in boiling 5 to 10 per cent. $AgNO_3$ solution for thirty seconds to five minutes to fix the cell contents and at the same time bring out the chloroplasts which take on a brown to black coloration.

Dilute aqueous methylen blue stains the cell-walls of algæ very readily. Cotton blue dissolved in Amann's lactophenol is useful for general structure (see Fungi). See also Davis, *Science*, lxxi, 1930, p. 16. Harris (*Watson's Micr. Rec.*, 1924 (3), p. 9) recommends Hofmann's blue after potassium permanganate and alum for freshwater algæ. Burton (*J. Queckett Micr. Club*, 2nd Ser., xv, 1923, p. 45) uses Hofmann's blue in 25 per cent. glycerin.

Chodat (*C. R. Soc. Phys. Hist. Nat. Genève*, xli, 1924, p. 140) and Deflandre (*Bull. Soc. Bot. France*, lxx, 1923, p. 738) use nigrosin; Curtis and Colley (*Am. J. Bot.*, ii, 1915, p. 89) picronigrosin. The cell-walls stain faintly, but the protoplasmic elements stand out in brilliant contrast against the black background. It is useful for most Myxophyceæ and some Chlorophyceæ.

Powers (see Chamberlain, p. 217) stains Volvocaceæ in Mayer's carmalum. See also Shaw, *Philippine Journ. Sci. Bot.*, xiii, 1918, p. 241. Margolena (*Stain Tech.*, vi, 1931, p. 47) uses Feulgen's stain for small forms. Methyl green in 1 per cent. acetic acid and Belling's iron aceto-carmine are most useful for living forms. See especially, Brand, *Arch. Protistenk.*, lii, 1925, p. 265.

Alcorn (*Stain Tech.*, x, 1935, p. 107) fixes desmids in 2 to 3 per cent. formaldehyde, to which a few drops of acetic acid have been added. Dehydrate, by decantation, through close alcohol grades (each 5 per cent. apart), about ten to fifteen minutes in each. Stain twelve to forty-eight hours in light green S.F. yellowish or fast green F.C.F. (1 per cent. in 95 per cent. alcohol). Wash in 95 per cent. and absolute alcohol, ten to fifteen minutes each. Clear in xylol series and mount in balsam.

1381. Taylor's Method for Sheath Structure of Desmids (*Trans. Amer. Microsc. Soc.*, xl, 1921, p. 94). Place fresh living material in 0·05 per cent. aqueous methylen blue for forty-five to sixty seconds. Remove, rinse in distilled water and place in one-tenth saturated aqueous picric acid. This fixes the stain

and brings out the striations in the sheath. Examine in the picric acid solution or remove after one to two minutes to water. The sheath begins to disintegrate in a few hours.

1382. Wall Structure of Heterokontæ, etc. Swell with strong caustic potash and stain with Congo red, or separate the portions by treatment with cold or warm 30 per cent. aqueous chromic acid.

For clearing use chloral hydrate when mounting in non-resinous media. Sodium hypochlorite is also good for dense parts, but its action is too violent for very delicate structures. Hydrogen peroxide is good for some very dense Phæophyceæ (HIGGIN's, *Ann. Bot.*, xlv, 1931, p. 345).

KUFFERATH (*C. R. Soc. Biol.*, xciv, p. 408) uses antiformin to reveal the structure of hard parts of algæ, the mixture being cleared by the addition of 10 to 25 per cent. acid. The method is especially useful with diatom frustules.

1383. Wall Structure of Bacillariales (Diatoms). The cell contents are removed by boiling in macerating liquids, especially mineral acids, or by heating on a slide to carbonise the cell contents. Delicate forms require more gentle treatment. Store frustules in 50 per cent. alcohol.

To Mount. Replace the alcohol with distilled water, shake and place a drop of material on a coverslip spreading it evenly. Allow the covers to dry and then heat to drive off any water in the frustules. Add a drop of thin xylol balsam on a slide and warm until firm. Special highly refractive media are often used. CONGER (*J. Roy. Micr. Soc.*, 1925, p. 43) uses styrax or piperine ($\mu = 1{\cdot}68$).

Dry Mounts. Prepare cells by making several superposed rings of cement on a slide. Invert a prepared coverslip, with frustules dried as above, while warm so that the edge of the slip lies on the cement ring. Press the cover slightly and evenly into the ring so that it adheres well. Allow to cool and give a coat of rather thick cement.

1384. Selected diatoms may be isolated from strewn covers by a mechanical finger attached to the microscope. The free tip of the finger is slightly greased to make it sticky. The diatom is redeposited on another coverslip in the centre of which is a thin smear of gelatin dissolved in glacial acetic acid. Gently breathe on the slide to cause the diatom to stick to the gelatin. The specimen may be manipulated as desired by the mechanical finger. Dry and mount as usual.

CONGER (*J. Roy. Micr. Soc.*, 1925, p. 43) finds that material is more easily withdrawn from a fine-grained, ground-glass slide rather than a polished one.

See also CABALLERO, *J. Roy. Micr. Soc.*, lxviii, 1927, p. 9.

MURRAY (*J. Roy. Micr. Soc.*, Lond., xlviii, 1928, p. 1) demon-

strates the form of diatom markings by impregnating the frustules with gelatin, staining with iron hæmatoxylin and mounting in 70 per cent. glycerin, which has the same refractive index as the diatom silica.

1385. Rhodophyceæ are difficult technically. The use of formalin (unless neutral and stored in the dark) should be avoided, especially with segmented forms, as it leads to disintegration. It is best to use chrom-acetic or formalin-acetic-alcohol for general fixation and preserve in 70 per cent. alcohol. Westbrook (*Ann. Bot.*, xlii, 1928, p. 149) also uses picro-uranium nitrate. Use the weaker Flemming solutions for filamentous forms and fix for short periods (a few minutes to one hour). Wash while dehydrating through the alcohols by close stages and keep in the dark. The large cœnocytes of some (*Griffithsia*) need special care.

Safranin and anilin blue give a good contrast; nuclei purple, chromatophores light blue and cell walls pink.

Section fleshy forms by freezing or imbedding in soap; paraffin often badly distorts them. Sturch (*Ann. Bot.*, xl, 1926, p. 585) cuts *Choreocolax* in frozen mucilage.

Calcareous species are especially troublesome. For general morphology and histology fix and decalcify in a large volume of chromo-acetic. Fix in a rather large volume of Flemming's for cytology and rapidly remove the CO_2 with a vacuum pump, adding additional chromo-acetic as the original becomes exhausted. Massive sorts, *e.g.* *Lithothamnion*, seem impossible.

1386. Philip's Method for Developing Procarps and Cystocarps (*Ann. Bot.*, ix, 1895, p. 303). Fix living material with chromo-acetic or aqueous iodine and place in 10 per cent. glycerin after staining, preferably with Hofmann's blue. A little eosin added to the glycerin will also stain the specimen. No stain is required in many cases. The walls of the procarps are gelatinised and swell greatly, so that the intercellular connections are readily visible.

Cryptonemiales. Split tips lengthwise exactly through the apex with a sharp razor. Lay both halves split side downwards on a coverslip, invert upon a slide and add water, tinted with eosin, from the side.

1387. Sporelings and Epiphytes. Culture on slides, fix and stain and mount in position. Use perfectly clean slides. If the organisms do not easily make a firm attachment, grind the slide slightly on one side with a sand blast or emery or etch with hydrofluoric acid.

Butcher (*Ann. Bot.*, xlvi, 1932, p. 813; *New Phyt.*, xxxi, p. 289) uses five glass slides 3 × 1 in. in a metal photographic frame secured to the river bed for the collection of algæ and the detection of sewage fungus (*Sphærotilus natans*). The slides are examined after four weeks.

For positively phototropic organisms (zoospores from *Chæto-phora*, *Œdogonium*, *Stigeoclonium*, *Ulva*, *Ulothrix*) place the

slide between them and the light source. For spores of Rhodophyceæ, other non-motile spores and eggs of Fucaceæ, distribute slides evenly over the bottom of the container. As soon as the material is attached, remove the slides to clean water and treat in a manner appropriate to the organisms studied. At intervals, remove slides and invert them flatly upon the surface of the fixative in fixing dishes and treat like a smear. Use fixatives and stains suitable for whole mounts of filamentous algæ.

1388. Charales. Handle like the delicate parts of phanerogams for imbedding, etc. Section branches longitudinally and axially through the reproductive organs. Safranin and anilin blue give a good contrast. Puncture the mature antheridium, stain whole and dissect before mounting. The mature oogonia are difficult to cut.

1389. Special Storage Products of Algæ. Leucosin (Chrysophyceæ) is soluble in most reagents and is unaffected by iodine. It dissolves in water after the cells have been fixed.

Paramylon (Euglenineæ) does not stain with iodine or chlor-zinc-iodide, but is soluble in concentrated sulphuric acid and potash. The grains swell and show a concentric stratification in dilute (6 per cent.) potash, finally dissolving. See Bütschli, *Arch. f. Protistenk.*, vii, 1906, p. 197; Molisch, *Mikrochem.*, 1923, p. 390.

Fucosan (Phæophyceæ) occurs in the form of vacuolar bodies, insoluble in water and unstained by iodine. It reduces osmic acid, the older vacuoles blackening especially rapidly. Fix with 25 per cent. HCl or H_2SO_4 and stain with methylen blue.

Floridean starch (Rhodophyceæ) occurs as small doubly refractive granules staining brownish or reddish with iodine.

FUNGI

1390. In general treat filamentous fungi like filamentous algæ and cut fleshy ones in paraffin. In the case of parasitic forms, however, the nature of the host tissues more often determines the technique than does the fungus. Many Xylariaceæ and other fungi become hard and brittle as they mature; break pieces off and tease them for examination. Cut sections of unimbedded material that has been steeped several weeks in equal parts of 95 per cent. alcohol and glycerin. Uredo- and teleutospores of Uredinales require treatment with hydrofluoric acid (1 part to 9 parts of water) to remove the silica; otherwise they cut badly.

Moreau (*Bull. Soc. Mycol.*, France, xxxiv, 1918, p. 137) is a compendium of mycological methods.

1391. Culture Methods. See Melhus (*Phytopath.*, ii, 1912, p. 197), for culture of parasitic fungi on living hosts.

Fungi (saprophytes) are usually cultured in Petri dishes or

flasks. The new Holden flask (HOLDEN, *Ann. Bot.*, xliv, 1935, p. 401) has several advantages; it largely obviates contamination and drying out of the medium and permits easy access to the colonies.

See SCHWEIZER, *Planta*, vii, 1929, p. 118, for a method of culturing coprophilous Ascomycetes cleanly; BODINE, *Science*, lxxiv, 1931, p. 341 (double plate method for *Tilletia*; CARLETON, *J. Appl. Micr. Lab. Meth.*, vi, 1903, p. 2109; CONN, *J. Bact.*, iii, 1918, p. 115).

1392. VERNON's Moist Chamber (*Ann. Bot.*, xlv, 1931, p. 733) is a useful device for observing sporangium formation, etc. Put a drop of agar culture medium on a slide. When it is cool to about the point of gelation, put a cover-glass on it and flatten out the drop of medium considerably. When the medium is hard, remove the cover-glass and cut the flattened drop through the middle, pushing one of the halves a little to one side, leaving a channel. Inoculate one of the halves along this side and replace the cover-glass. The aerial hyphæ and conidiophores growing into the channel are at right angles to the line of division.

See also BACHMANN, CIFFERI (§ 1394) and RIVALIER and SEYDEL (*Ann. Parasitol. Humaine et comp.*, x, 1932, p. 444) for methods of culture on slides.

The preparation of culture media is largely outside the scope of the present work. See RAWLINS, Chapter III; GWYNNE-VAUGHAN and BARNES, *The Fungi*; HARSHBERGER, *Mycology and Plant Pathology*, 1917, p. 581; LEVINE and SCHOELEIN, *A Compilation of Culture Media for the Cultivation of Micro-organisms*, Williams and Wilkins Co., Baltimore, Md., 1930.

1392 bis. Isolation of Single Spores or Bacteria. STOUGHTON (*A System of Bacteriology in Relation to Medicine*, H.M. Stationery Office, ix, 1931, p. 100) has summarised the more important methods. See also DAVIS (*Proc. Iowa Acad. Sci.*, xxxvii, 1930, p. 151).

1. Growth of a single selected cell in a smear on a suitable medium or in a dilution plate under direct microscopic observation, and its transplantation.

BURRI, *Das Tuschverfahren*, Jena, 1909; EZEKIEL, *Phytopath.*, xx, 1930, p. 583 (streak method); GARDNER, *J. Path. Bact.*, xxviii, 1925, p. 189; HORT, *J. Hyg.*, Camb., xviii, 1919, p. 361; LA RUE, *Bot. Gaz.*, lxx, 1920, p. 319 (dummy nosepiece); ØRSKOV, *J. Bact.*, i, 1922, p. 537; REIMANN, *J. Exp. Med.*, xli, 1925, p. 585.

2. Preparation of a hanging-drop containing a single organism which is then picked off by some mechanical device.

BARBER, *Philipp. J. Sci.*, ix, 1914, p. 307; CHAMBERS, *J. Infect. Dis.*, xxxi, 1922, p. 334; GREENE and GILBERT, *Science*, lxxv, 1932, p. 388; MALONE, *J. Path. Bact.*, xxii, 1918, p. 222; PETERFI, *Handb. biol. Arbeitsmethoden* (*Aberhalden*) *Abt.* v. *Teil* ii, 1924, p. 479; SCHOUTEN, *Verh. Akad. Wet. Amst.*, *Sect. Sci.*, xiii, 1911, p. 840.

3. Direct manipulation of the cell by (*a*) removing it on an agar surface to some other part of the surface;

DICKINSON, *Ann. Bot.*, xl, 1926, p. 273 (micro-isolator).

(*b*) lifting it bodily from a strewn preparation on a slide.

HANNA, *Ann. Bot.*, xxxviii, 1924, p. 701, and NEWTON, *Ann. Bot.*, xl, 1926, p. 109 (dry needle method); DUNN, *Phytopath*, xiv, 1924, p. 338 (micro-loop); EDGERTON, *Phytopath*, iv, 1914, p. 115.

4. Sterilisation of all cells except the selected one The selected cell is covered with a mercury droplet and the rest killed by ultra-violet light.

TOPLEY, BARNARD and WILSON, *J. Hyg.*, Camb., xx, 1921, p. 22; BARNARD, *Brit. J. Exp. Path.*, vi, 1925, p. 39.

5. Preparation of a dilute suspension in a capillary tube.

HANSEN (*Science*, lxiv, 1926, p. 384) breaks the tube into short lengths, selects those containing a single cell, sterilises them with alcohol and drops them into culture medium. GUPTA (*Brit. Myc. Soc. Trans.*, xix, 1935, p. 154) makes tiny droplets on a suitable surface by quick light touches and selects those containing a single cell only.

6. Single strains of fungi may be isolated by cutting out a hyphal tip and transplanting it to a suitable medium. Any suitable mechanical device can be used. See BROWN (*Ann. Bot.*, xxxviii, 1924, p. 401) who also describes a method for freeing fungal cultures from bacterial contamination; and MACHACEK (*Phytopath.*, xxiv, 1934, p. 301).

1393. For general fixation use absolute alcohol, saturated corrosive sublimate with 1 per cent. acetic in 95 per cent. alcohol, or Gilson's fluid, which is particularly good for fleshy forms.

OLTMANN (*Stain. Tech.*, x, 1935, p. 198) recommends a dilute (2 to 3 per cent.) solution of secondary butyl alcohol.

DUGGAR (*Fungous Diseases of Plants*, 1909) recommends a modified Gilson: 95 per cent. alcohol, 30 c.c.; glacial acetic acid, 2 c.c.; nitric acid, 5 c.c.; mercuric chloride, 10 grm.; distilled water, 270 c.c.

Follow the usual procedure after a mercuric fixative. CHAMBERLAIN (1932, p. 261) recommends hot (85° C.) corrosive sublimate-acetic acid (corrosive sublimate, 2 grm.; glacial acetic acid, 2 c.c.; and water, 100 c.c.). Fix for one minute. Wash in water and add iodine solution a few drops at a time, until it is no longer decolourised. Then stain.

See also: GILBERT, *Bull. Soc. Mycol. France*, xlv, 1929, p. 141 (iodine vapour).

EWART'S Method for the Preservation of Fleshy Fungi (*Ann. Bot.*, xlvii, 1933, p. 579). Soak material in a mixture of 2 parts of formaldehyde to 1 of liquid carbolic acid and after superficial drying suspend the specimen over strong ammonia until it sets solid without drying. The appearance is somewhat like that of

a candied fruit. The shape and structure are fully preserved, and by soaking in alcohol or water the whole of the impregnating material can be dissolved away. Impregnated specimens heated to over 100° C. are bakelised and become unaffected by water.

See also ULBRICH, *Zeits. Pilzkunde*, v, 1926, pp. 105 and 143, who gives details of the value of different antiseptics and of colour preservatives for particular kinds of fungi; and STOVER, *Trans. Ill. Acad. Sci.*, xxi, 1929, p. 187.

1394. Methods of Preparation. Many fungi, especially from cultures on solid media, are in the form of a loose mass of hyphæ. Removal of air is accomplished by flooding material on a slide with liquids of low surface tension, *e.g.* 0·5 per cent. gelatin or soap solutions or 70 per cent. alcohol. Mount for morphological examination in water, 3 per cent. acetic acid, 3 per cent. caustic potash or dilute chloral hydrate. Aqueous media of low refractive index are best. Use lactophenol (see also LINDER, *Science*, lxx, 1929, p. 430) or glycerin for stained material, or treat by the Venetian Turpentine or Balsam Infiltration Methods. Cytological details are best studied in material fixed with a Flemming fluid. WEBB (*Ann. Bot.*, xlix, 1935, p. 41) finds La Cour's 2B best for *Sorosphæra* (Plasmodiophorales). JONES (*Ann. Bot.*, xl, 1926, p. 607) and others use strong Flemming diluted with an equal volume of water. DUGGAR (*Fungous Diseases of Plants*, 1909) gives a special Flemming formula: 10 per cent. chromic acid 1·5 c.c.; 10 per cent. acetic acid 1 c.c.; 2 per cent. osmic acid in 2 per cent. chromic acid 5 c.c.; distilled water, 37·5 c.c. He prefers to bleach in bulk in 3 parts of 95 per cent. alcohol plus 1 part of hydrogen peroxide before imbedding. Filamentous types are extremely delicate, and must be handled very carefully through the alcohol grades and in imbedding. Duggar recommends the use of small dipper-shaped wire gauze ladles, the material being transferred undisturbed in the dipper.

Minute forms, such as yeasts and germinating spores may be handled in bulk. The material must be cultured in fluid media and killed in bulk, with a suitable fixative, depending upon the nature of the work intended. Wash in water or alcohol (depending upon the fixative employed) and grade into absolute alcohol. Place a drop of suspension on a coverslip and allow nearly to dry. Then flood the coverslip with water and allow the organisms to settle. Drain away the water and allow the cover to dry. Thorough drying should cause yeasts and other small structures to adhere. Then wet cover, stain and mount. Alternatively fix and then stain in Heidenhain's hæmatoxylin, dehydrate in glycerin by concentration and infiltrate with balsam.

HOOK and BRIGGS (*Science*, lxxx, 1934, p. 142) fix and stain germinating conidia of Peronosporales in iodine-potassium iodide solution (1 grm. iodine, 2 grm. KI and 300 c.c. water).

Green (*Ann. Bot.*, xli, 1927, p. 421) transfers *Zygorhynchus*, after fixing and washing, to 10 per cent. aqueous glycerin to which a drop of dilute acetic acid and 2 to 3 drops of saturated aqueous erythrosin have been added. Allow to concentrate in watch-glass and then mount in glycerin jelly.

Chamberlain (Methods, 1932, p. 261) gives a rapid glycerin mounting method for use with small forms. Fix two minutes in absolute alcohol. Stain two minutes in aqueous eosin solution and treat two to ten seconds in 1 per cent. acetic acid. Mount directly in 5 per cent. glycerin and seal. If the material collapses in the glycerin, put into 10 per cent. glycerin and allow to concentrate.

In the preparation of minute objects for sectioning, the use of a centrifuge is advantageous. Colley (*J. Agric. Res.*, xv, 1918, p. 619) describes a method for imbedding such material in paraffin while in the centrifuge.

Harper's method for handling such small structures is useful for many purposes, including the study of germinating spores and conidia. Make cultures in beerwort or other liquid medium on slides or in watch-glasses. Take up a drop of the suspension with a capillary tube and gently blow it out into a drop of weak Flemming fixative. Fix fifteen minutes to one hour or more. Smear a clean slide with Mayer's albumen. Draw up a drop of the material, without previous washing, into the capillary tube. Touch the tube quickly and lightly on the surface of the albumen, to leave minute dots of liquid containing material attached to the slide. Allow the fixative to evaporate somewhat, but do not let the preparation dry. Pass the slide rapidly through the alcohols to coagulate the albumen. Treat the slide now as though it carried sections.

Cultures of small forms (including bacteria) and germinating spores may also be treated as follows : (1) Spread a film on a slide, dry and fix by passing the slide a few times quickly through a flame, then stain ; or (2) spread a film on a slide previously smeared with albumen and allowed nearly to dry ; immediately invert flatly on fixing fluid in a fixing dish. Afterwards, wash and treat as a smear.

Bachmann's Method (*Amer. J. Bot.*, v, 1918, p. 32). Prepare clean sterile slides. Pour on to one slide a little of a culture medium (having an agar or gelatin base), inoculated with a suspension of the organisms to be examined. Place another slide on the first and draw them apart, leaving quite a thin film. Incubate the slides under sterile conditions in a damp chamber, *e.g.* in a Petri dish lined top and bottom with wet filter-paper. When the colonies are ready, fix and stain as for a smear. All media must be cleared for this method. She recommends potato broth agar for yeast.

Ciferri (*Mycologia*, xxi, 1929, p. 151) puts coverslips, coated with melted agar, singly into test-tubes containing a layer of cotton and filter-paper and autoclaves the tubes, which serve as moist chambers. Spores are sown or mycelium is planted on the coverslips. When grown, fix, stain, etc. The method is especially useful for Dermatomycetes; it is less successful with fungi having erect aerial conidiophores or sporodochia.

Overhots (*Proc. Int. Congr. Plant Sci.*, Ithaca, N.Y., ii, 1928, p. 1688) describes the technique of preparing mounts of Hymenomycetes.

See Boyce (*Phytopath.*, viii, 1918, p. 432) and Sinnott and Bailey (*Phytopath.*, xiv, 1924, p. 403) for methods of handling diseased wood.

Spores of fungi will adhere to slides if these are first smeared with Mayer's albumen. Invert the slides on a suitable fixative or dry and fix by heat Afterwards treat as a smear. For external structure mix with balsam or glycerin jelly and cover.

1395. Whetzel's **Method for Superficial Fungi** (*Journ. Mycology*, 1903). Strip off a piece of the epidermis with the superficial mycelium. Simmer over a low flame in 2 to 4 per cent. caustic potash for twenty to thirty minutes. Wash by standing in two to three changes of water for ten to twenty minutes each. Pick off the sub-epidermal tissue and if not clear again treat with hot caustic potash. Dehydrate in 95 per cent. alcohol, clear and mount. The host tissues are bleached, the dark hyphæ and reproductive bodies of the fungus not; the method is best for fungi with pigmented hyphæ. Staining could be attempted.

Abbott's **Method for Superficial Fungi** (*Phytopath.*, xv, 1925, p. 245). Dip a camel-hair brush into glycerin jelly and spread a thin coat on a warm slide. After the jelly has hardened, press the plant part bearing the superficial fungus against the jelly and remove it. A large part of the fungus is left in position in the glycerin jelly.

1396. Stains. Material examined directly may be stained on the slide with 0·5 per cent. aqueous eosin (followed by 2 per cent. acetic acid) or with alum-eosin (0·5 per cent. of each). Filaments difficult to stain will usually take Ziehl's carbol-fuchsin, as prepared for bacterial purposes. Material with coloured spores (Ascomycetes, Uredinales) may be counterstained with light green or Delafield's hæmatoxylin. Spores stain well with safranin. See also Hemmi and Endo, *Mem. Coll. Agr. Kyoto Imp. Univ.*, vii, 1928, p. 39.

Boss (*Dermatol. Wochenschr.*, xc, 1930, p. 482) treats a loop of filamentous fungi for two minutes on the slide with a mixture of 10 parts of 25 per cent. antiformin and 1 part of 1 per cent. caustic soda. He then fixes them with heat and stains them with Unna's modification of the Unna-Pappenheim stain.

Cotton blue (Baumwollblau 4B) or anilin blue in lacto-phenol are useful stains (see also § 1397). Maneval (*Stain Tech.*, xi, 1936, p. 9) describes the use of several stains with Amann's lacto-phenol or phenol glycerin. Fungi, algæ, etc., are well stained in lacto-phenol containing anilin blue, W.S. (cotton blue) or acid fuchsin, used singly or mixed. The addition of 20 to 25 per cent. glacial acetic acid makes staining more rapid and less deep. Delicate forms can be fixed and mounted in glycerin jelly, in which a small quantity of stain (*e.g.* gentian violet or safranin) has been dissolved. See also Davis, *Phytopath.*, xii, 1922, p. 492 (staining germinating spores).

For cytological details, staining in Heidenhain's hæmatoxylin and counterstaining with Congo red or with light green, erythrosin or orange G in clove oil has been most used. Newton's gentian violet-iodine method would probably be advantageous with most fungi. Gwynne-Vaughan and Barnes (*The Fungi*, 1927, p. 331) also suggests Flemming's triple stain or gentian violet and orange G alone; they state that if the nuclei are reasonably large Breinl's safranin-polychrome methylen blue-orange tannin combination is by far the best.

1396 *bis*. Staining Cell Constituents in Diseased Plant Tissues. The greatest changes in affected cells of diseased tissues are likely to occur in the vacuolar system.

Dufrénoy (*Stain Tech.*, iii, 1928, p. 57) therefore recommends fixation with Meves or Regaud fluids where embryonic cells, with dense cytoplasm, are to be studied cytologically in the same section with vacuolated cells. It is best to stain with acid fuchsin and destain with light green using Kull's method, preferably after Meves fixative. Post-staining with 0·5 per cent. aqueous toluidine blue and destaining with 0·5 per cent. aurantia in 70 per cent. alcohol give blue starch grains within red plastids. See also Dufrénoy, *Ann. Epiph.*, xiv, 1929, p. 227; Pelluet, *Ann. Bot.*, xlii, 1928, p. 637.

1397. Differential Staining of Parasite and Host. This is often difficult. The usual histological combinations are often successful. Gwynne-Vaughan and Barnes (*The Fungi*, 1927, p. 30) recommend safranin and light green and also methylen blue and erythrosin. These combinations are especially successful with hyphæ in the xylem of the host. If the host is woody, a lignin stain, preferably safranin or gentian violet, should be used, and various contrasting stains tested until one is found that suits the particular case. The following special methods may be employed.

Vaughan (*Ann. Missouri Bot. Gard.*, i, 1914, p. 241) uses the stain mixture Pianese IIIb, prepared from malachite green 0·5 grm., acid fuchsin 0·1 grm., martius yellow 0·01 grm., distilled water 150 c.c., 95 per cent. ethyl alcohol 50 c.c. Wash sections in water or alcohol and stain them in the mixture for fifteen to

forty-five minutes. Remove excess stain with water and differentiate in 95 per cent. alcohol, acidulated with a few drops of hydrochloric acid. Clear in carbol-turpentine or carbol-xylol, wash with xylol and mount in balsam.

To stain germinating spores on the epidermis of infected plants, prepare a leaf by placing a drop of spore suspension on the surface and keep it in suitable cultural conditions. Cut out test areas, fix them in equal parts of glacial acetic acid and 95 per cent. alcohol for twenty-four hours, wash in 50 to 70 per cent. alcohol, stain fifteen to thirty minutes in Pianese IIIb, wash for two minutes in water, hastily run through acid alcohol, dehydrate and clear as above.

Kobel (*Mitteil. Naturf. Ges. Bern.*, 1920, p. 44) stains the mycelium and haustoria of Peronosporaceæ and Uredinales in a solution of 0·1 grm. anilin blue in 50 c.c. of lactic acid and 100 c.c. of water. Stain sections for five minutes, rinse, and warm in a few drops of lactic acid. The mycelium is stained intensely blue while the host tissue remains nearly unchanged. The stain fades.

Cotton blue has frequently been advocated as a stain for fungal hyphæ. The stain may be dissolved in glycerin, lactic acid or lacto-phenol (Amann's medium) and the material either mounted in the coloured medium or after staining transferred to glycerin, lacto-phenol or glycerin jelly. When a solution of the stain is used as mounting medium it should be quite dilute. Staining is accelerated by gentle warming over a spirit flame.

Bright (*J. Roy. Micr. Soc.*, Lond., 1925, p. 141) examines mildewed cotton material by staining in picro-nigrosin or cotton blue, mounting in balsam, and examining the preparations under a low power using the powerful light source of a pointolite. The cotton fibres are rendered invisible and the fungal mycelia stand out clearly.

Chesters (*Ann. Bot.*, xlviii, 1934, p. 820) has described three methods for the use of cotton blue :—

1. For phycomycetes, sterilise a clean slide placed in the centre of a Petri dish and then fill the latter with about 30 c.c. of clear agar medium. This produces a thin film over the slide. Inoculate and incubate. Fix the mycelium in the agar for twenty-four hours at incubator temperature with 1 per cent. chromo-acetic acid. Remove slide with its attached agar film and wash thoroughly. Place slide in 10 per cent. glycerin and allow it to concentrate to the strength of Amann's medium. Stain six to twenty-four hours in 0·5 per cent. solution of cotton blue in Amann's medium and differentiate in several changes of this medium until no further colour is removed from the hyphæ. Wash out in 70 per cent. alcohol and carefully dehydrate to absolute alcohol. Pass through mixtures of alcohol and xylol

to pure xylol and infiltrate with xylol-balsam. The method can also be used for the early stages in formation of some perithecia and pycnidia.

2. For fungal hyphæ in woody tissues: stain thin sections in slightly warm 0·5 per cent. cotton blue in Amann's medium for five to fifteen minutes. Wash out excess stain with Amann's medium and remove this with 70 per cent. alcohol. Counterstain in safranin ten minutes and remove most of excess stain in 70 per cent. alcohol; dehydrate, clear in xylol and mount in balsam. Fungal hyphæ are stained blue, xylem red.

3. Examination of hyphæ upon cut surfaces of infected tissue (especially timber) may be carried out using a Leitz Ultropak vertical illuminator, after a pre-treatment of the surface with cotton blue. Smooth the surface with a sharp razor and immerse it in a slightly warmed 0·5 to 1·0 per cent. solution of cotton blue in Amann's medium for about ten minutes and wash away excess stain. Place block on slide and cover prepared surface with a coverslip.

LEPIK (*Phytopath.*, xviii, 1928, p. 869) obtains differential staining of Peronosporaceæ as follows: Remove paraffin from sections with xylol or turpentine; pass through absolute and 90 per cent. alcohols into a mixture (solution No. 1) of 10 grm. phenol, 10 c.c. conc. lactic acid, 20 c.c. conc. glycerin and 20 c.c. alcohol for ten to fifteen minutes. Then stain two hours in a mixture of 0·02 grm. cotton blue 4B (thought to be identical with oxanin blue 4BX or dianyl blue G) and 0·1 grm. safranin in 100 c.c. of solution No. 1. Differentiate in solution No. 1 and wash in absolute alcohol. Treat twenty to thirty minutes in a weak solution of safranin in clove oil and differentiate in clove oil. Clear in xylol and mount in balsam. Mycelium blue, host tissue red.

FERRARI (*Soc. Internag. Microbiol. Boll. Sez. Italiana*, iii, 1931, p. 26) recommends Cuccati's picro-carmine, which gives a rather intense rose colour. He also uses ruthenium red, which colours the cells red-violet. He finds that while a 10 to 20 per cent. aqueous solution of caustic potash bleaches the host cells, the mycelial cells are changed to a yellowish-red.

FERRARI (*Atti Ist. Bot.*, " Giovanni Briosi " *e Lab. Critogam. Italiano R. Univ. Pavia*, ii, 1930, p. 81). Immerse sections of host tissue in alcohol for ten to twenty minutes and then stain one to three minutes in a solution of 0·1 grm. ruthenium red in 15 c.c. distilled water. In obstinate cases stain the tissue twelve to twenty-four hours or heat the stain to boiling. Differentiate in a ten to twenty per cent. solution of caustic potash. The mycelium stains reddish-yellow, while the host tissue is destained. The stain must be kept in the dark.

DICKSON (*Science*, lii, 1920, p. 63) stains in a 2 per cent. solution of Magdala red in 85 per cent. alcohol for five to ten minutes,

removes excess stain in 95 per cent. alcohol and counterstains in 2 per cent. clove oil solution of light green for one to three minutes. He then washes in absolute alcohol or carbol-turpentine, clears in xylol and mounts in balsam. Parasite tissue stained red, host tissues green. If the tissues do not stain readily mordant with a freshly prepared 1 per cent. aqueous solution of potassium permanganate, rinse in water and pass up to 85 per cent. alcohol. CHAMBERLAIN (Methods, 1932, p. 273) suggests phloxine instead of Magdala red. DIEMER and GERRY (*Science*, liv, 1921, p. 629) suggest that fungal mycelia in woody tissues may be detected by taking advantage of the oxidising and reducing powers of the mycelium. They find that a silver nitrate solution stains the mycelium brown or orange and the wood lighter brown.

MOORE (*Science*, lxxvii, 1933, p. 23) recommends the use of pheno-safranin, prepared according to Satory's formula, viz., carbolic acid crystals 20 grm., lactic acid syrup 20 grm., glycerin 40 grm., distilled water 20 c.c., pheno-safranin 0·5 grm. or less. Mordant fixed sections with 2 per cent. iron alum for two hours. Stain and then differentiate with 0·5 per cent. iron alum in 0·5 per cent. HCl, or with alcohol. Intensify stain with 1 per cent. ammonia. The host tissue destains faster than the parasite.

CARTWRIGHT (*Ann. Bot.* xliii, 1929, p. 412) treats mycelium in wood sections as follows : Stain in 1 per cent. aqueous safranin ; it is usually sufficient to cover the section with stain and drain it off immediately. Wash in water, leaving a slight excess of stain. Cover the section with picro-anilin blue (25 c.c. saturated aqueous anilin blue and 100 c.c. saturated aqueous picric acid) and warm over a flame until the liquid is on the point of simmering. Wash out all the blue with water. Dehydrate, clear in clove oil, wash in xylol and mount in balsam. The mycelium is a clear blue, lignified walls red. Badly decayed wood takes up some blue, but the mycelium is clearly differentiated by its depth of stain.

STOUGHTON (*Ann. Applied Bot.*, xvii, 1930, p. 162) stained *Bacterium malvacearum* in diseased *Gossypium* with thionin and orange G. Stain for one hour in thionin solution (0·1 grm. thionin in a 5 per cent. solution of phenol in 100 c.c. of distilled water), dehydrate to absolute and counterstain in a saturated solution of orange G in absolute alcohol, for one minute. Wash thoroughly in absolute alcohol, clear and mount. Parasite, violet-purple ; cellulose walls, yellow or green ; xylem and chromosomes, blue ; spindle, purple. See MARGOLENA (*Stain Tech.*, vii, 1932, p. 25) for a modification.

See also BLACKMAN, *New Phyt.*, iv, 1905, p. 173 (Congo red for uredineæ) ; DURAND, *Phytopath.*, i, 1911, p. 129 ; HUBERT, *Phytopath.*, xii, 1922, p. 440 ; HADLEY, *J. Bact.*, ix, 1924, p. 405 (staining lytic areas produced by bacteriophage) ; RIDGEWAY, *Phytopath.*, vii, 1917, p. 389.

Mycorrhiza are often difficult. TAYLOR recommends, for roots of Orchidaceæ, fixation in chromacetic and heavy staining with safranin. Destain and counterstain in succession with methyl (or light) green and orange G. The older mycelium and host nuclei stain deep red, the younger mycelium pinkish or green, the host walls yellowish or red (if lignified).

MCLENNAN (*Ann. Bot.*, xl, 1926, p. 43) fixes *Lolium* roots with mycorrhiza in strong Flemming, stains in Heidenhain's hæmatoxylin and counterstains with eosin, or else stains in a fuchsin-iodine green combination. She states that roots cleared with carbol-alcohol and examined entire are useful in studying the distribution of the endophyte.

RAYNER (*Ann. Bot.*, xxix, 1915, p. 97) stained the mycelium in *Calluna* tissues with a strong solution of cotton blue in lactic acid for eight to twenty-four hours, differentiated in lactic acid and mounted in this reagent or in glycerin; the material could also be dehydrated and mounted in balsam. Strasburger's orseillin-anilin blue, iron hæmatoxylin and other common stains were also suitable for preparations of the leaves and shoot.

COHEN (*Stain Tech.*, x, 1935, p. 25) finds 5 per cent. chromic sulphate ($Cr_2(SO_4)_3 . 15H_2O$) in 4 per cent. formaldehyde (or 1 per cent. osmic acid), and saturated with picric or salicylic acids, is best for cytological details as well as proper fixation of the host tissue. Three per cent. acetic acid saturated with orseillin BB and 1 per cent. crystal violet in clove oil is a useful stain combination for infected plant tissue.

1398. Lichens are often hard to section when imbedded and are best cut unimbedded, using fresh or preserved material. Many of them, as well as the apothecia of Pezizales, etc., may be cut up small, stained in bulk with eosin, rinsed and teased out in 2 per cent. acetic acid. Or small pieces, stained with eosin, may be dehydrated, cleared in clove oil and teased before mounting in balsam. FRY (*Ann. Bot.*, xl, 1926, p. 397) imbeds corticolous lichens in paraffin after cutting away as much of the wood and secondary cortex as possible without breaking or stretching the bark. Carnoy is a good general fixative, stain in Heidenhain's hæmatoxylin with Congo red or erythrosin as counterstain, or in the cyanin-erythrosin combination. See also FRY, *Ann. Bot.*, xli, 1927, p. 437; *ibid.*, xlii, 1928, p. 141; PIERCE, *J. Appl. Micr.*, i, 1898, p. 99.

1399. COTNER'S **Method for Zoospores** (*Bot. Gaz.*, lxxxix, 1930, p. 295; *Amer. J. Bot.*, xvii, 1930, p. 511). Kill the zoospores in hanging drops on No. 1 coverslips, at the height of their activity, with osmic acid vapour from a 1 per cent. solution of osmic acid. Expose for fifteen seconds to two minutes; the longer exposure darkens the material, and the shorter one is preferable for details. Then add to the drop an equal volume of a 0·005 per

cent. solution of crystal violet. Allow the drop to evaporate to dryness at 22° to 24° C.; this takes eighteen to twenty-four hours. In humid climates the last stages must be carried out in a desiccator, allowing twenty-four to thirty-six hours over concentrated sulphuric acid. Now add clove oil with the least possible delay and differentiate under the microscope. Remove the oil with xylol and mount in balsam.

1400. Yeasts. WAGER (*Ann. Bot.*, xii, 1898, p. 449) first demonstrated the nucleus of Saccharomyces by the following method. Fix in a saturated aqueous solution of corrosive sublimate for at least twelve hours. Wash successively in water, 30 per cent. alcohol, 70 per cent. alcohol and methyl alcohol. Place a drop of the alcoholic suspension on a slide and, when nearly dry, add a drop of water. When the cells have settled, drain off the water and allow the preparation to dry. Place the slide in water for a few seconds and then stain with a mixture of fuchsin and methyl green, or fuchsin and methylen blue. Mount in glycerin or balsam. See also WAGER and PENISTON, *Ann. Bot.*, xxiv, 1910, p. 45.

Fixation of a smear in Flemming and staining with Heidenhain's hæmatoxylin gives good results.

KATER (*Biol. Bull.*, lii, 1927, p. 438) prepares films on slides previously smeared with albumen and fixes them immediately with corrosive sublimate-acetic-alcohol or preferably with Bouin's fluid. Stain with iron-alum hæmatoxylin with or without light green as a counterstain.

MANEVAL (*Stain Tech.*, iv, 1929, p. 21) states that in some kinds the nucleus may be demonstrated by fixing smears with heat, staining one minute with aqueous acid fuchsin, followed by 5 per cent. tannic acid for twenty seconds and a final washing with acidulated water. A mixture of equal parts of acid fuchsin and methyl green in water also is satisfactory. He also describes some of the most useful of Gutstein's methods (*Centrbl. f. Bakt.*, xciii, 1924, pp. 233 and 393; xciv, 1924, p. 145; xcv, 1925, p. 1; c, 1926, p. 1) of staining yeasts.

Gutstein's general procedure is: Fix smears by means of heat (three times over a flame) and stain two to three minutes with a 1 per cent. solution of a basic dye. Then wash with water and treat two minutes with a 5 per cent. aqueous solution of tannin, wash in water, counterstain, wash and examine.

The most useful combinations are: For vegetative cells of yeast: (1) Tannin followed by safranin; (2) carbol methylen blue, tannin, safranin; (3) methylen blue, tannin, safranin. For spores of yeast: (1) Carbol fuchsin, 5 per cent. acetic acid, tannin, safranin; (2) carbol methylen blue, 5 per cent. acetic acid, tannin, safranin. Maneval prefers to use 2 to 3 per cent. sulphuric acid in place of the acetic acid.

MANEVAL (*Bot. Gaz.*, lxxviii, 1924, p. 122) stains the spores with carbol fuchsin, sulphuric acid and methylen blue. He finds (*Stain Tech.*, iv, 1929, p. 21) that treatment of the preparation with tannic acid improves the staining of the ascus.

WEIDMAN and FREEMAN (*J. Amer. Med. Assoc.*, lxxxiii, 1924, p. 1163) emulsify yeast cells upon the slide with a drop of India ink.

KELLEY and SHOEMAKER (*Bot. Gaz.*, lxxxiii, 1927, p. 318) place a drop of dilute (1 : 10) Mayer's albumen on a slide and add a small drop of yeast culture and allow it to dry over a gentle heat. Stain 50 seconds in acid fuchsin, wash, dry and mount in balsam.

See also HENRICI, *J. Med. Res.*, xxx, 1914, p. 409.

Spore formation in yeasts can be secured by culturing on a moistened gypsum block (see WILL, *Centrbl. Bakt.* II, liii, 1920, p. 471).

1401. Myxomycetes. BROEKSMIT (*Nederland. Kruidk. Arch.*, 1925, p. 134) states that plasmodia and unripe sporangia should be gathered in small boxes lined with moist moss. The slightest damage causes cessation of development or abnormal growth.

HOWARD (*Amer. J. Bot.*, xviii, 1931, p. 624) collects plasmodia on plain agar in Petri dishes, and then repeatedly transfers them to plates of plain agar to free them of contaminants. Later they are grown on plates of rolled oat agar (30 grm. Quaker oats, 15 grm. agar, 1 litre of water) at 20° to 26° C.

See also AYERS, *J. Applied Microscopy*, i, 1898, p. 15.

Fix plasmodia with Flemming fluids ; if a plasmodium can be induced to creep on to a slide, it may be treated as a whole mount. Imbed in paraffin if separable from substratum ; if inseparable it is often better to imbed in celloidin.

GILBERT (*Amer. J. Bot.*, xxii, 1935, p. 52) fixes *Ceratiomyxa* in an alcoholic variant of Bouin :

Picric acid (saturated solution in 70 per cent. alcohol)	75 c.c.
Formalin (40 per cent.)	25 c.c.
Glacial acetic acid	5 c.c.

He employs smears to study mitosis in the spores and a modification of Cotner's method (§ 1399) for germinating spores and swarmers.

Stages in sporangia and spore formation require rather thin sections. Stain with Heidenhain's hæmatoxylin.

SKUPIENSKI (*Acta. Soc. Bot. Poloniæ*, vi, 1929, p. 203) grew *Didymium* under vital staining. It grew well in a mixture of equal parts 1 : 5000 neutral red and methylen blue. The neutral red stains the sporangia, the methylen blue the stipes.

BRYOPHYTA

1402. Mounts of whole plants and leaves, free-hand sections of thalli and leaves, fruits, spores and elaters, peristomes, etc., are

best made without staining in glycerin or glycerin jelly. Seal with damar balsam, etc. MURRAY (*Bryologist*, xxix, 1926, p. 55) puts sections and dissections on a slide into dilute glycerin and covers them. When the glycerin has concentrated by evaporation, remove the cover and as much of the glycerin as possible. Melt a little glycerin jelly on a coverslip, place it over the material and keep the cover in place with a small clip. Next heat the slide over a spirit flame until a distinct crack is heard. Cool, remove clip and leave in a formalin bath twenty-four hours. Clean slide and seal. See also BRUCH, *Ann. Bryol.*, vii, 1934, p. 6 ; CONARD, *Bryologist*, xxxvi, 1933, p. 2.

Peristomes may also be dried between two slides under a light pressure, moistened with xylol and mounted in balsam.

Skill in preparing free-hand sections is essential for the determination of species and the interpretation of anatomical features. HENRY (*Rev. Bryol.*, lii, 1925, p. 26) recommends that material to be sectioned in elder pith should first be imbedded in celloidin. For cytological studies fix in Flemming or, better, the mitochondrial fluids. Showalter uses Benda diluted with an equal volume of water or a modified Flemming (*Ann. Bot.*, xl., 1926, p. 713) : 200 c.c. 1 per cent. chromic acid, 12·5 c.c. 2 per cent. osmic acid, 3 or 6 c.c. glacial acetic acid and 215 c.c. distilled water.

Most Hepaticæ cut well in paraffin, but many Musci become brittle when prepared by the ordinary method. Use collodion or Zirkle's butyl-alcohol method.

1403. Culture. Sow spores on soil or, better, on a jelly medium to which nutrient salts are added. Knop's nutrient solution is satisfactory. MENGE (*Flora*, xxiv, 1930, p. 423) grew *Marchantia* and *Plagiochasma* on agar and silica jelly bases with the addition of nutrient salts. DERRING (*Verhandl. Naturhist. Ver. Preuss. Rheinlande u. Westfallens*, lxxxv, 1928, p. 306) cultivated *Buxbaumia* successfully on silica jelly with inorganic nutrients. For *Funaria*, SCHWEIZER (*Ber. Deuts. bot. Ges.*, xlviii, 1930, p. 75) used agar or sea-sand containing Detmer's solution, brought to the proper acidity by the addition of phosphoric, citric or huminic acids, or mixtures of them. See also CHALAUD, *C. R. Acad. Sci.*, Paris, clxxxiii, 1926, p. 612). ROBBINS (*Bot. Gaz*,. lxv, 1918, p. 543) finds that the addition of carbohydrates (levulose, mannose, glucose) to the medium favours growth, especially in the dark.

PTERIDOPHYTA

1404. The general histological and cytological methods are sufficient. Sections of root-tips and stem-tips, for apical cells and segmentation must be cut perfectly longitudinally and medianly. Fixation in chrom-acetic, followed by staining with Delafield's hæmatoxylin, gives good definition of these.

Lycopodiales. Leaves and strobili, particularly the older ones

in which the sporangia and spore walls have become hardened, are troublesome to cut in paraffin. Trim strobili flat on opposite sides to aid infiltration.

Equisetales. The stems, etc., are heavily impregnated with silica ; they must be desilicified and are best imbedded in celloidin. Growing tips are relatively free from silica and will cut in paraffin without special treatment.

Filicales. In fixing stem-tips, trim away the ramentum. Mature stems and roots of sclerenchymatous species should be treated as woody specimens. The walls of sporangia, particularly older ones, are resistant to fixatives, and for cytological purposes penetration must be assisted in every way ; Kihara's method (see § 1361) is satisfactory.

Fern Sorus. Examine fresh material with dark field illumination. Clear portions of leaves in KOH, wash and stain in safranin. Destain with care and lightly counterstain in dilute light green (or methyl blue) ; dehydrate, clear and mount in balsam.

Prothallia. Stokey (*Bot. Gaz.*, lxv, 1918, p. 97) grows fern prothallia on various media : different soil mixtures ; black peat, with and without Knop's solution ; porous clay crock standing in Knop's solution. Costello (Chamberlain, 1932, p. 314) grows them free from soil on a clean flower pot filled with *Sphagnum*, inverted in a dish of water, and covered with a bell jar.

See also Steil, *Bot. Gaz.*, lix, 1915, p. 254.

Flat ones are best fixed in medium Flemming and stained in Heidenhain's hæmatoxylin. To mount whole see § 1263. The gametophytes of heterosporous species surrounded by a hard impermeable spore wall should be fixed by Kihara's method (§ 1361 Carnoy followed by Flemming). Do not fix female gametophytes by this method after the megaspore wall has ruptured.

GYMNOSPERMÆ

1405. Careful treatment is required for megasporangial structures, the female gametophyte (endosperm) and the embryo. Segments should be cut as deeply as possible with a razor blade from opposite sides of the ovule to facilitate fixation. The ovule cuts well in paraffin until the stony layer is hardened. Free nuclear stages require special care to prevent shrinkage ; fix in Flemming with a higher proportion of osmic acid. For more accurate fixation, especially in *Cycadales* and *Ginkgo*, cut out the upper portion of the nucellus or endosperm. Secure pollen-tubes, with sperm mother-cells or sperms in their tips, in this way. Coulter and Chamberlain (*Morphology of Gymnosperms*) and Chamberlain (*Methods in Plant Histology*) give data on the times of attainment of various stages in development of the different organs in several species.

The female cones of Coniferæ should be dismembered for all

except the youngest stages. Individual cone scales are best cut back to the ovule, when the tips become hard. In older stages, remove the ovules from the scale and trim them as far as possible.

LUTZ (*Stain Tech.*, vi, 1931, p. 123) describes a method for preparing thick sections of mature pine cones. Thoroughly air-dry the cone and imbed in paraffin wax of melting point 56° to 58° C. Cooling must be quick and complete. Mark lines of sections on the paraffin and cut section with either a hacksaw or carpenter's handsaw. Dress down the surface with sand-paper and, if required, coat the polished surface with white shellac.

1406. BUCHHOLZ's **Method for Pinus Embryo** (*Bot. Gaz.*, lxvi, 1918, p. 185). Dissect the embryo from living material under a 0·3 grm. molar sugar solution (about 10·3 per cent.). Isolate the gametophyte first and hold it gently with forceps by the broad end and make a cut right round the narrow end with a needle shaped to an arrow-head tip and keenly sharpened. Gently remove this end, and by teasing into the end of the ovule expose the rosette ends of the suspensors, which should be pushed out by the straightening suspensors. By successive segmental cuts the distal portions of the suspensors and the embryo, are exposed, and the whole complex removed. Fix in formol alcohol, and stain in Delafield's hæmatoxylin, the embryo being handled by means of wide-tipped pipettes. Dehydrate through glycerin and infiltrate with Venetian turpentine or balsam.

ANGIOSPERMÆ

1407. Pollen Morphology. WODEHOUSE (*Journ. New York Bot. Gard.*, xxvii, 1926, p. 145) recommends that grains be examined both dry and moist. The latter condition reveals more characters to advantage. WODEHOUSE (*Ann. Bot.*, xlii, 1928, p. 891) gives methods for preparation of microscopic mounts and (*Bull. Torrey Bot. Club*, lx, 1933, p. 417) for catching and counting atmospheric pollen. He recommends an alcohol-water-glycerin combination for temporary preparations, and Brandt's glycerin jelly for permanent ones. For a stain he uses aqueous methyl blue, since it stains the exine selectively; it fades in eight to nine months. The procedure is: Take a little pollen on the tip of a scalpel, place on a slide, moisten with a drop of 95 per cent. alcohol and stir with a needle to a paste, which rapidly dries, leaving the pollen lightly stuck to the slide. Wash by flowing alcohol over the pollen and drawing off with filter-paper. When nearly dry add a drop of methyl blue and warm to hasten staining; draw off excess with filter-paper. Add a little melted glycerin jelly and cover. Allow jelly to set with slide inverted, so that grains may be close to the cover.

He also details the treatment of herbarium specimens. Moisten

anthers with 95 per cent. alcohol, followed by a large drop of distilled water. Heat slide until water boils, open anthers and press out the pollen. Stain and pass into glycerin or glycerin jelly. To mount dry or shrunken material, stain in a drop of anilin oil, moderately tinted with gentian violet. Heat over a flame until the oil steams ; if necessary continue for several minutes to secure a good stain. Dry off part of the oil and add a drop of balsam.

MARGOLENA (*Stain Tech.*, ix, 1934, p. 71) fixes in Bouin and stains in 0·5 per cent. aqueous solution of Bismarck brown. Rinse in water, dehydrate with 95 per cent. absolute alcohols. Counterstain and differentiate with about 0·3 per cent. fast green FCF in clove oil. Clear in xylol. Exine green, intine brown, nuclei brown. FERGUSON and COOLIDGE (*Amer. J. Bot.*, xix, 1932, p. 644) consider that descriptions of pollen grains observed in aqueous media are of doubtful value. They find that grains of *Petunia* do not change appreciably in size and shape when passed directly from dry air to xylol balsam, where they remain permanently unchanged.

CHAMBERLAIN recommends that loose pollen of anemophilous plants should be soaked a few minutes (fifteen to twenty) in water before fixation in bulk and handling like minute organisms.

1408. Pollen counts to determine percentages of normal and abnormal or empty grains are made from covered mounts in a drop of iodine solution or in 45 per cent. acetic acid lightly coloured with iodine. Care should be taken to sample the preparation thoroughly as the smaller and empty shrivelled grains tend to collect at the edges of the coverslip. Make measurements soon after mounting the pollen as they will swell (and burst) or sometimes shrink quite rapidly, often in thirty to sixty minutes.

See BLAKESLEE and CARTLEDGE, *Proc. Nat. Acad. Sci.*, U.S.A., xii, 1926, p. 315.

1409. Pollen Germination. Most pollens rapidly lose their vitality, so that tests should not be delayed. Some grow well on slides kept in a damp chamber. Others require water ; allow the drop to spread thinly on a clean slide so that oxygen is readily available. Most will germinate in a solution of sucrose, the optimum concentration of which varies with the species, and from year to year, over a wide range. DOROSHENKO (*Bull. Appl. Bot., Genet. and Plant-Breed.*, xviii, 1928, p. 217) gives a table for over 500 spp., as to the optimum conditions for the germination of their pollen *in vitro*, for its storage and for its longevity..

TRANKOWSKY'S **Method** (*Planta*, xii, 1930, p. 1). Slides are spread with a thin coating of agar (1 to 2 per cent.) containing sugar in suitable concentration, dusted with pollen and placed in a moist chamber. After germination the slide may be fixed, stained and mounted in balsam.

See also PODDUBNAJA-ARNOLDI, *Planta*, xix, 1933, p. 299.

1410. Pollen Tubes in Style. Pistils should be artificially pollinated and collected after an interval. They should be collected in small phials and kept from drying out by the addition of a drop of water. Slender styles and ovaries may be crushed and fixed on the slide ; larger ones are first sectioned longitudinally by hand. Styles fixed whole should be split longitudinally to permit easier freeing from air with a vacuum pump. A dilute solution of anilin blue stains the pollen-tube walls readily.

Buchholz and Blakeslee (*Science*, xl, 1922) scald styles of *Datura* in hot (not boiling) water for two minutes, slit lengthwise and dissect away the cortical tissue. Stain the central core in magenta (acid red), wash a little in water, clear in lactic acid, mount whole in concentrated lactic acid and seal. Pressure applied to the coverslip spreads the material in a thin layer. The pollen-tubes show as dark red streaks among the elongated pink cells of the conducting tissues. The stain improves in twelve to twenty-four hours.

Buchholz (*Stain Tech.*, vi, 1931, p. 13) has further developed the technique. Artificially pollinated pistils are kept at 18° to 22° C. Styles are split along two sides, scalded half to two minutes in water at 70° to 75° C. and killed by immersion several hours in 50 per cent. alcohol containing 6 per cent. of formalin. Within twelve hours of killing, dissect the cortex from the style and stigma under a wide field binocular microscope. Stain the central strand within twenty-four hours of dissection in a mixture of 8 parts 1 per cent. aqueous acid fuchsin and 2 parts 1 per cent. aqueous alcoholic light green for three to six hours or overnight if stain not too concentrated. Clear several hours in 80 per cent. lactic acid, carefully spread out on slide and mount in lactic acid. Seal after a few days with xylol damar or paraffin wax and gum mastic.

See also Buchholz and Blakeslee, *Mem. Hort. Soc.*, N.Y., iii, 1927, p. 245.

1411. Chandler's Aceto-carmine and Magenta Method (*Stain Tech.* vi, 1931, p. 25). Pistils are collected and immediately killed in a mixture of 6 to 7 c.c. formalin in 100 c.c. 70 per cent. alcohol. They may be examined at once or stored. Those with a central canal are split longitudinally with needles along one side to the central canal and the cut surfaces spread apart. Solid styles are sectioned longitudinally freehand. Place a drop of aceto-carmine (saturated solution in warm 45 per cent. acetic acid) on the exposed surfaces. After a few seconds, add a drop of saturated aqueous magenta. Remove excess stain with blotting paper. Destain by passing absolute alcohol over the style and absorbing it at the basal end with blotting paper. If tissue differentiation is difficult, add a drop of anilin blue just before the magenta.

1412. NEBEL'S **Lacmoid-Martius Yellow Method** (*Stàin Tech.*, vi, 1931, p. 27). Crushed or sectioned fresh material is stained in a mixture of 5 mgm. lacmoid and 5 mgm. martius yellow dissolved in 10 to 15 c.c. water, adjusted to *p*H 8 by the addition of a few drops of 1 per cent. ammonia, thus making the fluid deep olive-green. After two to five minutes the material is mounted in the stain or in water of the same *p*H. To make permanent mounts fix crushed, dissected or otherwise prepared material in Carnoy and transfer through 70 per cent. alcohol to alkaline water. Wash thoroughly in tap-water to remove all acid or excessive alkali. Stain one to five minutes and dehydrate rapidly in alcohols containing lacmoid in solution in its blue modification (*p*H 8). Mount in cedar oil and seal, or in balsam after clearing with xylol.

1413. WATKIN'S **Cotton Blue-Lacto-phenol Method** (*Journ. Genet.*, xv, 1925, p. 340). Originally applied to wheat, the technique is applicable to all Gramineæ. Note a flower being self-fertilised, take the spike into the laboratory, place it in water and leave for one hour. Dissect out the feathery style and stain and mount in a 0·08 to 0·1 per cent. solution of cotton blue in lacto-phenol (equal parts of lactic acid, phenol, glycerin and water). Over-staining begins in one to four months; stronger solutions are unsatisfactory.

AIYANGAR (*Agric. and Live-stock in India*, i, 1931, p. 471) uses lacto-phenol for studying pollen-tube entry into the cotton ovule.

CHAPTER LIII

PALEOBOTANY

1414. General accounts of paleobotanical methods are given by:

Krausel, *Die palaobotanischen Untersuchungsmethoden.* Jena, 1929.

Hofmann, *Mikrokosmos* (Stuttgart), xxiii, 1930, p. 93.

Jurasky, *Aberhalden, Handb. biol. Arbeitsmethoden*, Abt. xi, 1931, p. 253 (section methods) and p. 331 (maceration methods).

Potonié, *Zeits. Bot.*, xiii, 1920, p. 79 (maceration and staining).

Netolitzky, *Mikrokosmos* (Stuttgart), xx, 1927, p. 178 (methods for carbonised material).

1415. Maceration Methods. See the papers given above, especially those by Potonié and Jurasky. The most successful macerating fluids are Diaphanol (from Leitz), nitric acid and aqua regia. They also have the effect of bleaching the material and must be removed subsequently by thorough washing with water. Staining can sometimes be attempted; try safranin or hæmatoxylin in the first instance.

1416. Sectioning Methods. Formerly most of the harder fossils were sectioned by grinding, following geological methods. Cut thin slabs through the specimen in the desired direction with a hack-saw or band-saw of the type used for cutting mineral specimens. The blade must be of the hardest temper. The operator of a band-saw must be thoroughly protected. Then grind the specimen as thinly as possible while being held in the hand, and polish one surface. Cement the polished surface to a glass slide. When firmly attached complete the grinding and polishing, wash, dry with alcohol, clear if necessary and cover with balsam and a cover-glass. The details of the method depend very largely upon the matrix. Exercise caution when the section is approaching the desired thinness to ensure an even thickness and to prevent it breaking up. Some friable specimens should be saturated with resin before grinding. Hoskins (*Bot. Gaz.*, lxxxix, 1930, p. 414) describes a transfer method for sections. Lomax (*Journ. Roy. Micr. Soc.*, xlvii, 1927, p. 239) describes the technique of making coal sections. First coat the block with shellac to prevent absorption of moisture and then grind the end level, heat to remove moisture and coat with shellac. Next heat the block in an oven and then cool. Smooth the ground end with fine carborundum on a glass plate, and again shellac and dry the

surface. Apply a thin layer of clove oil to the smooth surface and press it, either cold or slightly warm, on a heated glass slide, using a mixture of balsam and gum copal for cement. Cool the block and leave it until it is firmly cemented to the slide. Place it in a vice and cut it through with a hack-saw, leaving a thin slice fastened to the slide. Grind this slice to the desired thickness, dry and add balsam and a coverslip. SEYLER and EDWARDS (*The Microscopical Examination of Coal*, H.M. Stationery Office, London) give methods for cutting thin sections and a method, based on metallographic practice, of polishing and etching flat surfaces to bring the plant structures into prominence. TURNER (*Amer. Inst. Min. and Met. Eng. Advance Paper*, No. 1409–1, 1925) polishes small blocks of anthracite, etches them with heat and examines them with reflected light using a metallographic microscope. HEARD (*Quart. J. Geol. Soc.*, lxxxiii, 1927, p. 195) imbedded pyritised specimens, from the Old Red Sandstone, in shellac and carefully ground and polished them with the finest carborundum flour. The polished sections were then treated with concentrated nitric acid to bring out the internal structure of the plants.

The differentiation of cell-wall and other details in fossilised specimens usually depends on the presence of carbonised material representing the plant, imbedded in some matrix, usually siliceous or calcareous. Sections in which there is insufficient differentiation may be much improved by KISSER's Anthracogram method (*J. Indian Bot. Soc.*, x, 1931, p. 60). Pass them through alcohol and benzol and boil them in liquid paraffin under a large coverglass for one minute or more. Traces of organic matter in the walls are carbonised and appear first yellowish, then reddish-brown and finally blackish. When the desired degree of carbonisation has been reached, cool the slide, dissolve away the paraffin and mount in the usual way.

Semi-fossilised peats, soft brown coals and partially silicified woods may be desilicified with hydrofluoric acid and sectioned on a sliding microtome with or without imbedding in celloidin. LANG (*Ann. Bot.*, xliii, 1929, p. 663) dehydrates and imbeds in paraffin wax from chloroform; sections can then be obtained in ribbons.

JEFFREY (*Anatomy of Woody Plants*) recommends that the material be soaked in carbolic acid under pressure in a wire-stoppered bottle in a paraffin oven both before and after treatment with hydrofluoric acid. The periods of immersion in each reagent should be about one week, and the treatments should be repeated if necessary.

Peel Methods of Sectioning Petrifications were originally due to WALTON (*Nature*, cxxii, 1928, p. 571); see also *Nature*, cxxv, 1930, p. 1413. Grind a flat surface on the petrified mass, parallel

to the plane from which the section is desired. Immerse this surface for a time in hydrochloric acid (coal balls and other calcareous masses) to dissolve away the carbonates. Find the concentration of the acid and the duration of the etching process by trial. Wash with water and flood the etched surface with a liquid (*e.g.* the trade preparation Durofix) which forms a tough film on drying. When dry peel off the film, which will carry with it a thin section of the fossil. Wash in acid and in water, dry clear and mount in Canada balsam under a coverslip. The thickness of the section depends upon the time of action of and the strength of the acid used in etching. Virtually serial sections may be obtained. For silicified material, hydrofluoric acid replaces the hydrochloric acid used for etching.

KOOPMAN'S (*Geol. Bur. Netherlands*, Heerlen, cxxxi–cxxxii, 1929) uses a celluloid solution. He moistens the surface with amyl acetate to avoid air bubbles. BARNES and DUERDEN (*New Phyt.*, xxix, 1930, p. 74) remove bubbles from the drying film by pouring ether fumes on them and polish the lower surface of the film with fine knife powder. The transfer is washed and well smeared with Mayer's albumen. A slide is warmed and the transfer is well pressed on to the slide; flattening and adhesion is aided by a few drops of absolute alcohol. The slide is then placed nearly vertically in equal parts of absolute alcohol and ether to remove the celluloid.

DUERDEN (*Ann. Bot.*, xlv, 1931, p. 376) adds a plasticiser (castor oil, triacetin, or benzyl abietate) to the cellulose acetate or cellulose nitrate (pyroxylin) solution, to avoid puckering of the sections due to unequal drying. The following mixtures are good: (1) Cellulose nitrate in equal parts of alcohol and ether, plus 5 per cent. of castor oil; (2) cellulose nitrate in 2 parts of acetone and 1 part of amyl acetate, plus 2 per cent. triacetin; (3) cellulose acetate in 4 parts of acetone and 1 part of diacetone alcohol, plus 1 per cent. each of benzyl abietate and triacetin. A mixture of the solvents is poured on the surface of the rock before pouring the film. The film mixtures should be of the consistency of pure glycerin.

GRAHAM (*Stain Tech.*, viii, 1933, p. 65) finds a most satisfactory film is given by 20-second nitrocellulose 20 grm., butylacetate 200 c.c., tricresyl phosphate or methyl phthalate about 1 c.c., toluene or xylol 10 to 20 c.c. It dries in four to six hours, but is best left one to two days.

See also KRICK, *Bot. Gaz.*, xciii, 1932, p. 153.

1417. Impressions and Incrustations. WALTON'S **Canada Balsam Transfer Method** (*Ann. Bot.*, xxxvii, 1923, p. 379). Clean the visible surface of the material and set it on a glass slide with hard to brittle balsam. Imbed the mount in paraffin, leaving only the remnants of the excess of rock matrix exposed. Dissolve these

away with hydrofluoric acid. The preparation, after washing, can be kept dry and examined by reflected light, or mounted in glycerin jelly under a coverslip. It cannot be mounted in balsam. The method makes possible an examination of both surfaces of carbonised plants. It is a development of the collodion film method of Nathorst (*Geol. Foren Forhandl.*, xxix, 1907 ; see also BATHER, *Geol. Mag.*, Dec. v, iv, 1907).

Ashby's Cellulose-Film Transfer Method (LANG, *Ann. Bot.* xl, 1926, p. 710). Treat the exposed surface with a solution of cellulose acetate in amyl acetate (or celloidin solution, or the trade preparation "necol") and allow the surface to dry thoroughly. If necessary, repeat the treatment to obtain a strong film. Grind away the superfluous rock, and place the specimen in hydrofluoric acid in a wax vessel until the cellulose film is free and clear of mineral matter. Wash in water, dehydrate in 95 per cent. alcohol (absolute alcohol must not be used). Clear in terpinol, oil of bergamot, etc. (clove oil must not be used), and mount in Canada balsam, applying slight pressure with a clip if necessary.

See also THOMAS, *Phil. Trans. Roy. Soc. Lond.* B., ccxiii, 1925, p. 299 ; BOLTON, *Ann. Bot.*, xliii, 1929, p. 414.

The transfers may be examined by reflected light, a Leitz-Wetzler vertical illuminator being useful in conjunction with high powers (Walton). They are commonly rather opaque to transmitted light. Infra-red photography (WALTON, *Nature*, cxxxv, 1935, p. 265) yields very detailed pictures. DIXON (*Nature*, cxxxv, 1935, p. 958) uses an Ultropak microscope with coal material.

1418. Fossil Pollen. A summary of the methods at present in use is given by GODWIN (*New Phyt.*, xxxiii, 1934, p. 278). Peat samples are treated with hot alkali, calcareous marls with hydrochloric acid, and the organic matter of the siliceous sediments is obtained by decantation and treatment with hydrofluoric acid.

For peat samples boil up a small amount with a few drops of 10 per cent. caustic potash or caustic soda on a slide. Examine the macerated material directly, or wash and mount in glycerin jelly, either unstained or tinted with safranin. Modifications involve using known weights or volumes of peat, repeated washings, centrifuging and making up in such a manner that a known amount is present on each slide. Pressure on the coverslip causes over-representation of large pollen grains, by squeezing out smaller ones around the edge.

ERDTMANN's method (*Svensk. Bot. Tids.*, xxvii, 1933) makes it possible to examine samples too poor in pollen for the alkali method. Mildly oxidise the sample with $NaClO_3$ in a mixture of acetic acid and sulphuric acid for some hours in the cold. Wash the residue and dry it with acetone and ether. Then treat with 80 per cent. sulphuric acid, which hydrolyses the polysaccharide

fraction, leaving a sample rich in pollen grains. Make up the residue with lacto-phenol stained with methylen blue, and place a definite volume for counting in a specially made chamber of the type of a blood-corpuscle counter.

1419. See also DOCTUROVSKY, *Reports on Peat*, iii, 1914 (Russian, French summary); RUDOLPH and FIRBAS, *Ber. Deutsch. Bot. Ges.*, xl, 1922, p. 393; STARK, *Zeits. Bot.*, xvii, 1925, p. 89; GAMS, *Zeits. Gletscherkunde*, xv, 1927, p. 161 (who gives a full bibliography); SEARS, *Bot. Gaz.*, lxxxix, 1930, p. 95.

CHAPTER LIV

A GUIDE FOR STUDENTS OF MICROTOMY

1420. *Three Examples for Beginners* :—(1) The preparation of *whole stained mounts* of some small object (Daphnids).

(2) The preparation of *sections of the muscle or an organ of a vertebrate.*

(3) The *preparation of an embryo* (or tadpole) for the making of serial sections.

Example I. From a pond or ditch obtain some water-fleas (*Daphnia* or *Simocephalus*) ; allow the jar to stand for several hours till the suspended material has settled. Capture some of the organisms as follows :—Take a piece of glass tubing some 8 inches in length ; place a finger over one end, dip the other end under the water and by taking away the finger, suck up some of the Daphnids into the tube ; put your finger over the end of the tube, remove the latter and transfer the organisms to a capsule or watch-glass about 2 inches in diameter. With a clean pipette carefully suck up most of the water, hardly allowing the animals enough to swim in ; now add a fixative to kill the organisms (see § 10), this to coagulate their protoplasm (§ 28) as rapidly as possible so as to leave the groups of cells forming the organs intact and *in situ.*

Use corrosive acetic acid (§ 68), 2 per cent. acetic acid in saturated aqueous corrosive. Pour the fixative into the watch-glass or capsule, till it is full (the watch-glass or capsule contains about 15 to 20 c.c.). Place a glass square or plate over the capsule, and leave it for thirty minutes. *The organisms become opaque,* indicating the coagulation of the protoplasm of their cells.

With a pipette carefully remove as much of the fixing fluid as possible. Now that the organisms are killed, the mercury salt must be removed ; *unless the fixative is thoroughly removed, it will form masses of pin-shaped crystals at a later stage* when the animals are being mounted in balsam.

To remove the corrosive sublimate, it is necessary to convert it into another substance which may be more easily washed away; this is effected by immersing the animals in some 70 per cent. alcohol which has been coloured light port-wine shade with tincture of iodine (§ 68), whereupon the mercury bichloride becomes mercury iodide, which is very soluble in 70 per cent. alcohol. The iodine and alcohol mixture should be used until it no longer loses its colour, which indicates excess of iodine. The whole process should last several hours and may be carried on overnight.

The iodine and 70 per cent. alcohol are poured away, and the animals washed for several hours (a minimum of two) in at least two changes of 70 per cent. alcohol to remove as much of the iodine as possible. The objects are then transferred to 50 per cent. alcohol for one half-hour, then into 30 per cent., for the same time. They are brought down these grades in order that shrinkage may not occur when they are being transferred to stains containing little alcohol, or none at all.

Two stains may be tried, Mayer's acid hæmalum (§§ 288 and 289), and Grenacher's alcoholic borax carmine (§ 271). The time that

both these stains should be used depends almost entirely upon the accessibility of the cells of the object to the stain. Daphnids are covered by a chitinous shell, which though delicate tends to prevent instant penetration. It is a good thing to leave the animals in the stain for about five hours at least, and overnight preferably.

Take two clean capsules, pour into one about 10 c.c. of borax carmine, into the other a similar quantity of the hæmalum. With a camel-hair brush or a pipette transfer some of the organisms to the stains and leave as directed above. *See that the capsules are securely covered.*

After some hours in the stain, the latter is poured back, and the process of differentiation (§ 240) is begun. The object of differentiation is to wash away superfluous stain from certain organs or parts of organs, in order that a contrast in depth of colour may be obtained in the various other organs and tissues. Both borax carmine and Mayer's acid hæmalum may be differentiated in acid alcohol (4 to 6 drops of HCl to 100 c.c. of 70 per cent. alcohol), which should generally be allowed to act at least as long as the stain has been used, and, if necessary, longer. In both cases when differentiation has reached the right stage, the objects examined under a microscope have a transparent appearance, *and such parts as the viscera and muscles should be well contrasted.*

The borax carmine specimens are washed out for several hours in neutral 70 per cent. alcohol. They are then upgraded to 90 per cent. and absolute alcohol, two hours in each, or overnight in absolute alcohol, and cleared in methyl benzoate, cedar-wood or clove oil for at least two hours, and then mounted in xylol balsam.

The hæmalum specimens have to be brought to an alkaline solution in order to " blue " the stain, and to get rid of all acid. Some workers " blue " the stain in 70 per cent. alcohol made slightly alkaline with ammonia or bicarbonate of soda, but the best results are obtained by downgrading the objects to tap-water, which is allowed to run over them gently till they go quite blue, which should occur for small objects within an hour. The animals are then gradually upgraded through 30, 50, 70 and 90 per cent., to absolute alcohol and cleared as above described for borax carmine specimens.

In order to obviate the differentiation stage, one may dilute both the borax carmine and the acid hæmalum till they are about one-third or one-half as strong ; dilution of the borax carmine may be carried out with 50 per cent. alcohol (not methylated spirit) and with distilled water in the case of hæmalum. In these solutions the animals remain till sufficiently stained. But the best results are got by the overstaining and differentiation method.

1421. *Example II.* From a frog remove a large leg or thigh muscle, and cut it into two pieces about as big as the nail of the little finger. If desired, the liver, a halved testis, or a kidney may also be used.

Transfer the material to a capsule containing at least 20 c.c. of Susa (wash out in 90 per cent., see § 68), Zenker's or Helly's fluid (§ 78). Leave till next morning, and wash in running water under the tap for at least three hours, preferably overnight, then transfer to 50 per cent. alcohol for an hour ; then to 70 per cent. alcohol, containing enough tincture of iodine to give the solution a light port-wine shade. Add more iodine as the colour disappears, prolonging the treatment overnight for large pieces. Pour away the alcohol, and add pure 70 per cent., in which the material is washed at least three hours. Transfer to 90 per cent. for several hours and leave in absolute alcohol overnight. Next morning it is safest to give the material another hour in a fresh change of alcohol absolute. Pour away a good deal of the alcohol and

add about the same quantity of xylol or cedar oil. Shake, leave half an hour, and then transfer the material to pure xylol or cedarwood oil; leave half an hour. Pour away some of the xylol, either add chips of hard wax to cover the tissue, or add some of the stock xylol and wax mixture. Leave an hour in thermostat on the upper shelf, pour off, and add molten pure wax; leave one or two hours on the bottom shelf. Imbed blocks (§§ 156, 157).

1422. *Example III. Preparation of an Embryo for Serial Sections.* Fix in Bouin's fluid, corrosive acetic or picro-nitric, overnight (§§ 115, 68, 102). In the case of the first and last mentioned fixatives, the embryo is afterwards transferred to 30 per cent. alcohol (half-hour), 50 per cent. (two hours), and then washed for a day in several changes of 70 per cent. The corrosive acetic fixed specimens are similarly treated except that at this stage iodine solution is added to the 70 per cent. (or this may be done in 90 per cent.) alcohol till the corrosive sublimate is removed. Leave overnight in 90 per cent. alcohol (or at least three or four hours), and at least six hours in two changes of absolute alcohol (preferably overnight). De-alcoholisation and clearing must be done carefully as directed in § 134, p. 68. It is a good plan to bring embryos from absolute alcohol, through several gradually strengthening mixtures of alcohol and cedar-wood oil—to pure cedar-wood oil, and then wash out in benzol. Imbed in wax as described in § 158, generally about one hour in benzol and wax, and two hours in pure wax. Imbed blocks (§§ 156, 157). Now read §§ 174 *et seq.*

1423. General Rules and Hints for Students. (1) Keep all your bottles and capsules as clean as possible.

(2) Try to keep your bench in order.

(3) Keep notes of the time necessary for changing reagents.

(4) Thoroughly clean your slides and coverslips in acid alcohol before using. See addendum.

(5) Note that corrosive sublimate tends to harden material.

(6) Corrosive sublimate is difficult to remove from tissue unless you use iodine. If not properly removed you will find numerous pin-shaped crystals in the finished sections. § 68.

(7) Corrosive sublimate attacks the surface of steel and other metals. Use quills or wooden needles for manipulating tissue in sublimate.

(8) Watery stains after picric acid fixation will cause maceration if prolonged. § 98.

(9) Unless very well washed out, picric acid should not be used in conjunction with thionin or toluidin blue. Precipitates form. Certain other dyes do likewise.

(10) Osmic acid crystals should be dissolved in the purest distilled water. Wash the tube with distilled water before you break it, removing label. Wash out capsules and bottles for osmic acid solutions in distilled water. Keep solutions in shade or dark. § 40.

(11) Osmic acid tends to harden yolk and certain other cell materials. The vapour of osmic acid is injurious to the eyes and nose.

(12) Osmic acid and fixatives containing it inhibit staining, but if necessary you can induce osmicated material to stain in delicate dyes by bringing sections down to distilled water and treating in a 0·25 per cent. solution of permanganate of potash for a short time. Permanganate also decolourises sections. See p. 309 and § 701.

(13) Nitric acid tends to soften chitin and yolk, but it may inhibit staining a little. § 102.

(14) Imbed material in paraffin in the shortest time possible, for materials left in the thermostat longer than necessary go hard, especially from xylol; this refers especially to vertebrate material and yolky embryos.

1424. General Plan of Procedure Applicable to Histological Specimens.

Anæsthetise animal, kill it, quickly take out organ, cut pieces 1 cm. × 1 cm. × ½ cm.

↓

Fix for 24 hours in Zenker's fluid $\left\{\begin{array}{l} 2\tfrac{1}{2}\% \ K_2Cr_2O_7 \\ 5 \ \% \ HgCl_2 \\ 5 \ \% \ \text{Gl. Acetic acid.} \end{array}\right.$

↓

Washing in running water 24 hours.

Freezing Method.

Preserve in 5% Formalin
↓
Wash in water
↓
Cut sections with freezing microtome
↓
Float in water on to slide
↓
Stain with Picro-carmine 15 minutes
Drain off and wipe away the stain around
↓
Mount in Farrants' medium.

Paraffin Method.

Pass through increasing strengths of alcohol
↓
70% alcohol
↓
Remove Hg deposit with iodine in 70% alcohol
↓
(Preserve in 70 % alcohol)

Stain
↓

Pass through 90% alcohol....................1 day
Dehydrate in abs. alc..................... 1 night
↓
Clear in xylol or benzol......From 1 to 2 hours
↓
Pass through xylol satd. with paraffin wax 1 hour
↓
Impregnate with paraffin at 52° C.....2 hours
and
Imbed and make blocks
↓
Cut sections with microtome
↓
1° Apply glycerin and albumen water to slide
↓
2° Float section on slide
Warm gently to open out the section
Wipe away excess of water and dry in warmth overnight
↓
3° Remove paraffin with xylol
↓
4° Remove xylol with abs. alc.
↓
5° Pass through 90 and 70% alcohol to water
↓
6° Stain in hæmatoxylin, etc., 5–15 minutes
↓
7° Wash in water, 5 minutes—1 hour
↓
8° Counterstain in eosin—1 minute
↓
9° Remove excess with 90% alcohol
↓
10° Dehydrate with abs. alc.
11° Clear in xylol
12° Mount in balsam.

Celloidin method.

↓
Alc-ether aa ....1 day
↓
Thin celloidin.........................1 week
(15% in alc-ether)
↓
Thick celloidin..........................1 week
(30% in alc-ether)
↓
After evaporation, mount on block of vulcanised fibre.
↓
Harden celloidin in chloroform........1–2 hours
and then in 80% alc.....................1–6 hours
↓
Cut with razor (oblique) wetted with 80% alc.
↓
Stain without removing celloidin
↓
(Remove celloidin with oil of cloves)
↓
Mount in balsam.

(Modified from D. T. Harris' "Practical Histology.")

(15) Alcohol and chloroform dissolve fats and lipoids, acetic acid dissolves lipins. Vegetable oils dissolve fats less readily than xylol or chloroform. Read §§ 133 *et seq.*

(16) Strong alcohol is bad for the finger nails and skin.

(17) When diluting stains with alcohol, use solutions made up by breaking down pure absolute alcohol. Do not use methylated spirit, as this generally precipitates the stain.

(18) You can soon learn to tell roughly the strength of alcohols by the smell.

(19) Don't use the dregs of the absolute alcohol bottle for dehydrating anything. The dregs are no longer absolute. Keep a waste alcohol bottle for used liquid.

(20) Some workers add a little bag of fused copper sulphate to their store bottles of absolute. This keeps the alcohol dehydrated.

(21) After fixation, when dehydrating and imbedding a piece of tissue, an egg or an embryo, it is at its softest when in weak alcohol, and its hardest when in xylol or a clearing oil. Flatten or otherwise manipulate a fixed object, while it is still in weak alcohol, or it will break up; but some objects may be dissected successfully in clove oil. § 8.

(22) Cells alter soon after death: formalin fixation is the best for corpse material. Carefully note § 33.

(23) The organs of animals over-anæsthetised by chloroform or ether are often spoilt (especially in the vicinity of large blood-vessels) and are sometimes useless even for general purposes.

(24) Keep balsam or colophonium jar in the dark, or paint it black outside. Acid balsam soon removes stains from tissue; acid balsam is the microtomists' *bête noire.* §§ 483 and 499.

(25) After Zenker fixation sections may overstain in eosin.

(26) If finished sections have crystals in them this is due to improper washing out of fixative, or stain.

(27) Formaldehyde gas dissolves in water up to 40 per cent. The commercial formalin is acid and must be neutralised. § 113.

(28) Formaldehyde gas is injurious to the skin and mucous membrane of nose.

(29) If after staining in delicate dyes (*e.g.*, methyl green), all the colour keeps coming out of the sections during passage through alcohols, try the following method:—Wipe superfluous water from around the sections, and dehydrate by dropping acetone on sections: then plunge into a jar of half acetone, half xylol, then pure xylol.

(30) For clearing embryos or pieces of tissue for whole mounts, chloroform, carbon bisulphide, or cedar-wood oil are better than xylol. § 133.

(31) If bubbles get under the coverslip they can often be removed by gently warming, or by placing slide under bell jar of an exhaust pump.

(32) If after mounting an object in balsam white or black lines and blotchy areas appear, this means that dehydration was not complete. Bring back through xylol to absolute alcohol.

(33) When, after imbedding, the block is set aside for a time and it is found that the object is surrounded by a halo of white wax, this means that all the clearing oil was not removed and is now exuding from the object. Re-imbed in pure wax.

(34) When, after imbedding, the material seems soft and tends to fall out of the wax, this indicates that dehydration was not complete, and possibly also that the time in pure wax was not long enough. Without efficient dehydration it is impossible to make good sections.

(35) If when cutting the sections curl up, it means that either the knife is blunt or the material has been overhardened during imbedding. Occasionally an incorrect slope of the knife may be the cause of curling.

(36) When the sections will not form a ribbon, this means that either

the wax is too hard or the slope of the knife is not correct. If the wax is hard, place 1 drop of soft wax on each side of the block and flatten it out with a warm knife. Read carefully pp. 83 to 96.

(37) The broad side of a block should be parallel to the knife.

(38) Some people use miniature drums for rolling up the wax ribbon. Laying them on a piece of foolscap does quite well. Avoid sticky paper. If sections accidentally adhere you can often release them by cautiously wetting the paper with 90 per cent. alcohol.

(39) Before placing sections on a slide, write with a diamond pencil the number of the slide and the material used. At a pinch, a glass wax-pencil may be used instead.

(40) If you have not used a diamond, it is always possible to tell on which side of the slide the section lies, simply by slightly tilting the slide and observing the shadow thrown on the other side of the glass.

(41) For fixing and imbedding hard material read §§ 172 to 177.

(42) Dioxan technique is much used nowadays. Dioxan is a cumulative poison and should only be used in well-ventilated rooms.

(43) For imbedding very small objects read § 157.

(44) For making preparations of insects read methods in § 1188.

APPENDIX I

1425. Cleaning Slides and Covers. *New* ones should first be soaked in one of the following liquids : strong sulphuric, hydrochloric or nitric acid, or *aqua regia*, or a mixture of an ounce each of sulphuric acid and bichromate of potash with from 8 to 12 ounces of water, then washed first with water and lastly with alcohol, and dried with a clean cloth.

For *used* ones, if a balsam mount, warm, push the cover into a vessel with xylol or other solvent of the mount, and put the slide into another vessel with the same, leave for a few days and then put into strong alcohol. If this is not sufficient, treat as for new ones. Some persons boil in lysol, which we do not find efficacious.

For the final treatment, see § 208.

For chemically free vessels, see § 779.

1426. Gum for Labels. Labels stuck on glass often strip off. This may be avoided (MARPMANN, *Zeit. Angew. Mik.*, ii, 1896, p. 151 ; *Journ. Roy. Mic. Soc.*, 1897, p. 84) by means of the following adhesive : 120 grm. of gum arabic are dissolved in a quarter of a litre of water, and 30 grm. of gum tragacanth in a similar quantity. After a few hours the tragacanth solution is shaken until it froths, and mixed with the gum arabic solution. Strain through linen and add 150 grm. of glycerin previously mixed with $2\frac{1}{2}$ grm. of oil of thyme.

1427. Marking Slides Instead of Labels. For many years, quick-drying varnishes, etc., have been used for marking slides. Transparent lacquer is advocated by Philip Smith, who paints the end portion of the slide with "Luc," allows to dry, writes the title, and covers the writing with another coat (WATSON's *Microscope Record*, No. 16, 1929). Similar paint can be made by dissolving celluloid in amyl acetate.

PEIRCE (*Journ. app. Mic.*, ii, 1899, p. 627 ; *Journ. Roy. Mic. Soc.*, 1900, p. 404) finds that if the end of the slide be painted with a thin solution of balsam, it may be written on with ink when dry, and the record preserved by a second coat painted over it.

For other receipts see *early editions*.

1428. J. BAKER's **Method for Pressing Down a Coverslip.** While a balsam or glycerin-jelly mount is hardening, place the slide flat and put a small cork (from a specimen tube) on the middle of the coverslip. Put enough pennies on the cork to give

sufficient pressure. Two pennies suffice for most purposes. This method avoids the messiness often associated with the use of spring clips, and the pressure can be much more accurately adjusted to requirements.

1429. How to Remove Coverslips. It is often desirable to remove the coverslip from an old preparation in order to restain the sections or to replace a cracked cover. The safest plan is to place such a slide in a jar of xylol and put it aside until the cover loosens. Sometimes the addition of a little absolute alcohol to the xylol helps to dissolve away the damar or balsam. CARLSON (*Science*, lxxxi, 1935, p. 365) recommends a mixture of 9 parts xylol and 1 part of *n*-butyl alcohol, since it works much more rapidly than xylol alone. *Caution*—*n*-butyl alcohol will dissolve anilin dyes and should not be used if this matters.

The quickest method of removing coverslips is to warm the slide cautiously over a small flame and pull the cover off with the fingers ! If done skilfully, few, if any, of the the sections will be disturbed, but the method is not recommended for valuable slides.

1429 *bis*. Tap Water, Tap Water Substitute, Alkaline Water. Some tap waters are alkaline enough to "blue" hæmatoxylin, and to wash traces of acid out of stained sections and smears. Others are not suitable, and in that case you keep a tap water substitute, which is merely distilled, or more or less neutral tap water with 0·2% to 0·5% sodium bicarbonate added. This is washed off with distilled water. S. G. Scott's tap water substitute is $KHCO_3$, 2 gm., $MgSO_4$, 20 gm., Aq. dest. 1,000 c.c., with a crystal of thymol to prevent growth of moulds. Tap water substitute is not intended for washing out after fixations. It would be unsuitable for this purpose.

APPENDIX II

VARIOUS SALT SOLUTIONS

1430. Physiological Saline Solution

Frog	0·64% NaCl.	Selachians 1·5 to 2·6% NaCl according to species (Rodin).
Salamander . .	0·80% ,,	
Warm-blooded animals	0·90% ,,	

Ringer Solution

For Amphibians

NaCl	0·65 grm.	
KCl	0·025 ,,	
$CaCl_2$	0·03 ,,	
[$NaHCO_3$ . . .	0·02 ,,	] *p*H7·0–7·4 (*circa*)
Aqua dest. . . .	100 c.c.	

For Warm-blooded Animals

NaCl	0·85 grm.
KCl	0·025 ,,
$CaCl_2$	0·03 ,,
Aqua dest. . . .	100 c.c.

N.B. If the solution is used with $NaHCO_3$, although this is not the general practice, the calcium chloride must be added last to the solution, in order to avoid the precipitation of insoluble calcium carbonate.

The solution does not keep well, and should be preferably freshly made on each occasion.

Ringer-Locke

NaCl	0·85 grm.	(For cold-blooded animals 0·65 grm. NaCl.)
KCl	0·042 ,,	
$CaCl_2$	0·025 ,,	
$NaHCO_3$ . . .	0·02 ,,	
Aqua dest. . . .	100 c.c.	

The remarks *re* addition of calcium chloride in the making up of the last solution apply equally to this.

Locke-Lewis solution is exactly similar to the above save that it contains 0·01 to 0·25 grm. dextrose.

Since this solution should also be freshly made just before use, it is convenient to keep the following solutions always made up.

NaCl	9%
KCl	1%
$CaCl_2$	1%
$NaHCO_3$	10%

Immediately before use 20 c.c. NaCl, 4 c.c. KCl, 4 c.c. $CaCl_2$ are added to 200 c.c. aqua dest. If sterility is desired, the above solution is heated to boiling. *After cooling*, 0·4 c.c. $NaHCO_3$ is added.

N.B. A solution containing $NaHCO_3$ must *on no account* be heated, since by loss of CO_2 the bicarbonate becomes transformed into the carbonate. If desired, it may be filtered through a Berkefeld filter.

Tyrode

NaCl	0·8 grm.	
KCl	0·02 ,,	
$CaCl_2$	0·02 ,,	
$MgCl_2$	0·01 ,,	
NaH_2PO_4	0·005 ,,	
$NaHCO_3$	0·1 ,,	*p*H 7·5–7·8 (*circa*)
Grape sugar	0·1 ,,	
Aqua dest.	100 c.c.	

This solution may not be boiled. If desired, it may be sterilised through a Berkefeld filter.

Fleisch solution is a modified form of Tyrode, and may be made up in two stock solutions, which may be boiled without harm.

Stock Solution I

NaCl	8·0 grm.
KCl	0·2 ,,
Normal Na_2CO_3 sol.	20 c.c.
Aqua dest.	500 ,,

Stock Solution II

$CaCl_2$	0·2 grm.
$MgCl_2$	0·1 ,,
N. HCl sol.	8·0 c.c.
N. H_3PO_4 sol.	3·5 ,,
Dextrose	1·0 grm.
Aqua dest.	500 c.c.

Just before use equal parts of the two solutions (after cooling) are taken, and *Sol. I. is added to Sol. II.* The prepared solution (I. and II.) must not be boiled. *p*H of solution = 7·52.

Hédon-Fleig Saline

NaCl	0·7 grm.
KCl	0·03 ,,
$CaCl_2$	0·01 ,,
$NaHCO_3$	0·15 ,,
Na_2HPO_4	0·05 ,,
$MgSO_4$	0·03 ,,
Glucose	0·1 ,,
Aqua dest.	100 c.c.

*p*H *circa* 8·8

The above solution is extremely useful for teasing out, etc., pieces of tissue from animals with a body fluid of high *p*H (*e.g. Helix aspersa*, *p*H 8·4).

To preserve the active movement of mammalian sperms for a long time suspend them in this solution :—

Baker's Buffered Glucose-Saline

Glucose	3·0 grm.
$Na_2HPO_4 . 12H_2O$	0·6 ,,
Sodium chloride	0·2 ,,
KH_2PO_4	0·01 ,,
Distilled water	100 c.c.

(See J. R. Baker, *Quart. Journ. Exp. Physiol.*, xxi, 1931, p. 139.)

See also §§ 621, 780, 1120.

APPENDIX III

PETER GRAY'S BASAL SOLUTIONS FOR LABORATORY FIXATIVES *

1431. FIXATIVE solutions are often required in small quantities. Their preparation from stable basal solutions (GRAY, *Journ. R. Microsc. Soc.*, liii, 1933) prevents waste of time, material and storage space. Ten aqueous and two alcoholic solutions are required :—

(1) 80% nitric acid.
(2) 40% formaldehyde.
(3) Glacial acetic acid.
(4) 2% chromic acid.
(5) 7·5% potassium dichromate.
(6) " Müller × 3 " (7·5% potassium dichromate plus 3% sodium sulphate).
(7) 7% mercuric chloride.
(8) Saturated picric acid.
(9) 1% "platinic chloride" ($PtNa_2Cl_6$).
(10) 2% osmic acid.
(11) 1% picric acid in 95% alcohol.
(12) 1% each of picric acid and mercuric chloride in 95% alcohol.

Notes. Aqueous solutions (1) to (9) and both alcoholic solutions are stable, and of constant content, at temperatures above 12° C. Solutions (5), (6) and (7) start throwing down crystals if dropped below this point. Solution (10) is stable only in chemically and mechanically clean bottles in the presence of from 0·01 to 0·05 per cent. potassium permanganate; this concentration, which is easily judged colorimetrically, must be maintained by periodic additions of a stronger solution. Such osmic solutions have retained their strength for three years. (*P.G.*)

Solution (5) may be substituted for solution (6) by those who do not believe in the efficacy of the sulphate content of Müller. GRAY (*loc. cit.*) states that it minimises shrinkage and collapsed cavities in mammalian embryos.

* By Peter Gray.

1432. Table for the Preparation of Aqueous Fixatives from Basal Fixative Solutions. Prepared by Peter Gray

FIXATIVE.	Basal Fixative Solutions.										Water.	95 % Alcohol.	Other Additions.
	1	2	3	4	5	6	7	8	9	10			
	80% HNO_3.	Formalin.	Glacial Acetic.	2% CrO_3.	7½% $K_2Cr_2O_7$.	Müller × 3.	7% $HgCl_2$.	Sat. Sol. Picric Acid.	1% Pt. Na_2Cl_6.	2% OsO_4.			
ALTMANN	—	—	—	—	30	—	—	—	—	50	20	—	—
APÁTHY	15	30	—	—	—	—	—	—	—	—	65	—	—
BENDA	—	—	2	—	10	—	—	—	—	20	65	—	—
BENSLEY	—	11	—	—	—	30	70	—	—	—	—	—	—
BOUIN	—	25	5	—	—	—	—	75	—	—	—	—	—
BOUIN-McCLUNG	—	25	5	—	—	—	—	75	—	—	—	—	1 gram urea
BOVERI	—	—	1	—	—	—	—	35	—	—	65	—	—
BURCKHARDT	—	—	3	30	20	—	—	—	—	—	45	—	—
CARLETON	—	10	—	—	—	—	90	—	—	—	—	—	—
CHAMPY	—	—	—	20	16	—	—	—	—	22	33	—	—
ERLICKI	—	—	—	—	33	—	—	—	—	—	67	—	1 gram $CuSO_4$
FLEMMING	—	—	—	—	—	—	—	—	—	—	—	—	—
1. Chrome acetic	—	—	0·1	12·5	—	—	—	—	—	—	88	—	—
2. " Weak "	—	—	0·1	12·5	—	—	—	—	—	5	83	—	—
3. " Strong "	—	—	5	38	—	—	—	—	—	20	38	—	—
4. " Without acetic "	—	—	—	40	—	—	—	—	—	21	40	—	—
FOL	—	—	—	13	—	—	—	10	—	—	78	—	—
GATENBY	7	—	—	25	13	—	—	—	—	—	60	—	—
GERHARDT	—	25	2·5	13	—	—	25	—	—	—	35	—	—
GILSON	1·5	—	0·4	—	—	—	35	—	—	—	50	7	—
HELLY	—	5	—	—	—	30	70	—	—	—	—	—	—
HERMANN	—	—	5	—	—	—	—	—	38	2	38	—	—
HILL	4·75	—	—	—	—	—	—	95	—	2	—	—	—
HOYER	—	—	—	—	27	—	30	—	—	—	44	—	—
KAHLE	—	12	2	—	—	—	—	—	—	—	60	30	—
KLEINENBERG	—	—	—	—	—	—	—	100	—	—	—	—	2 c.c. H_2SO_4

FIXATIVE.	Basal Fixative Solutions. 1. 80% HNO_3.	2. Formalin.	3. Glacial Acetic.	4. 2% CrO_3.	5. $7\frac{1}{2}$% $K_2Cr_2O_7$.	6. Müller × 3.	7. 7% $HgCl_2$.	8. Sat. Sol. Picric Acid.	9. 1% Pt. Na_2Cl_6.	10. 2% OsO_4.	Water.	95% Alcohol.	Other Additions.
KOHN	—	—	5	—	70	—	20	—	—	—	10	—	—
KOLATCHEV	—	—	—	20	—	18	—	—	—	20	42	—	—
KOSTANECKI, 1	4	—	—	—	—	—	50	—	—	—	46	—	—
„ 2	4	—	—	—	—	—	33	—	—	—	29	33	—
KULSCHITZKY	—	—	2	—	27	—	4	—	—	—	21	50	—
LAVDOWSKI, 1	—	—	1	—	—	50	2	—	—	—	45	—	—
„ 2	—	10	2	—	—	—	—	—	—	—	60	30	—
LENHOSSÉK	—	—	2·5	—	—	—	40	—	25	—	35	—	—
LINDSAY-JOHNSON	—	—	5	—	22	—	—	—	7·5	10	56	—	—
MANN	—	—	—	—	—	—	50	—	—	25	25	—	0·75 gms. NaCl.
MAXIMOW, 1	—	9	—	—	—	30	—	—	—	—	60	—	—
„ 2	—	10	—	—	—	30	—	—	—	11	60	—	—
MAYER	5	—	—	—	—	—	—	100	—	—	—	—	—
MERKEL	—	—	—	7	—	—	—	—	7	—	90	—	—
MÜLLER	—	—	—	—	—	30	—	—	—	—	60	—	—
ORTH	—	—	—	—	—	33	—	10	—	—	66	—	—
PERÉNYI	5	—	—	7·5	—	—	—	—	—	—	59	30	—
RABL—													
1. Picro-sublim.	—	—	—	—	—	—	25	25	—	—	50	—	—
2. Plat. sublim.	—	—	—	—	—	—	25	—	12·5	—	63	—	—
3. Plat. picric.	—	—	—	—	—	—	—	20	10	—	75	—	—
VOM RATH	—	—	1	—	—	—	—	100	—	6	—	—	—
RAWITZ	1	—	—	40	—	—	—	20	—	—	40	—	—
REGAUD	—	20	—	—	32	—	—	—	—	—	48	—	—
SCHAUDINN	—	—	5	—	—	—	66	—	—	—	—	33	—
SMITH	—	5	2·5	—	13	—	—	—	—	—	80	—	—
TELLYESNICKY	—	—	5	—	40	—	—	—	—	—	60	—	—
ZENKER	—	—	5	—	—	30	70	—	—	—	—	—	—

1433. Table for the Preparation of Alcoholic Fixatives from Basal Fixative Solutions

Prepared by Peter Gray

FIXATIVE.	1	2	3	11	12	Chloro-form.	Water.	Absolute Alcohol.	Other Additions.
Van Beneden	—	—	50	—	—	—	—	50	—
Carnoy, 1	—	—	25	—	—	—	—	75	—
,, 2	—	—	30	—	—	30	—	30	—
Carnoy-Lebrun	—	—	30	—	—	30	—	30	Saturate with $HgCl_2$
Carnoy-Sanson	—	—	5	—	—	30	—	65	Ditto.
Duboscq-Brasil	—	26	6·5	45	—	—	22	—	—
Olmacher	—	—	5	—	—	15	—	80	Saturate with $HgCl_2$
Petrunkevitch	2	—	17	—	—	—	50	38	Ditto.
Yocum	—	5	20	—	55	—	—	—	20 c.c. ether.

TREATMENT OF CUTS, ETC.

By Dr. R. H. Micks and Mr. Louis Werner

Small clean cuts need no treatment to help them to heal. Bleeding can be stopped quickly by holding a wisp of clean cotton-wool to the cut for about five minutes; when the bleeding has stopped the cotton-wool may be allowed to remain stuck to the cut, and can be secured by a loose bandage. If the cut is so large that the edges gape widely it will usually have to be stitched by a surgeon.

Avoid the application of finger-stalls and collodion dressings, for they delay healing by preventing evaporation of water from the skin. If it is necessary to protect a cut from irritants a finger-stall may be slipped on for a short time, but it should be removed as soon as the need for it has passed.

Fainting occurs very readily in some people with even trivial injuries. The proper treatment for a faint is to place the patient flat on the floor; no other treatment is necessary.

Infected material is rarely handled by the microtomist, but should any injury to the skin occur when working with fresh animal tissues medical advice should be sought without delay.

Irritant gases may cause serious damage to the lungs, and it should be remembered that the first signs of damage may not occur till several hours after exposure. If irritant gases have escaped into a laboratory it must be evacuated at once and not entered again until it has been declared safe.

Carbon monoxide poisoning does not damage the lungs, but to continue working in a laboratory in which an escape of gas has occurred is very dangerous. An early sign of poisoning may be impaired power of reasoning, and a man who has been working for some time in an atmosphere vitiated by carbon monoxide is often incapable of appreciating the risk he is running and may have to be removed against his will.

Injury to the eye may occur either from fluids or from solid particles.

The first essential in cases of splashes of liquids is immediate irrigation. Time should not be wasted hunting around for a suitable neutralising solution of alkaline or acid reaction, but the affected eye should be held open (fingers on the eyelids will be needed) in a basin of water, and if possible moved around while under water. If an assistant is present he can carry out the irrigation with water.

Having washed away as much irritant as possible, oil should be instilled, castor oil, cod-liver oil, or even olive oil, but *not* cedar-wood oil. These measures suffice for the time that elapses before skilled medical attention can be obtained. No time should be

lost in securing this, no matter how well the subject feels, and no attempt should be made to resume work if any irritation is felt. A solution of cocaine is not likely to be at hand, but, if it is, a drop or two will relieve a lot of the immediate pain and facilitate subsequent examination.

Minute solid particles tend to lodge under the upper lid, so that if the upper lid is seized by the lashes and drawn forwards and downwards over the lower lid the skin surface of the lower lid may serve to wipe away foreign material. If the particle is still felt in the eye after this manœuvre, the best thing to do is to take the old-fashioned advice and rub the *other* eye. This evokes a free flow of tears in both eyes, and this flow may sweep a foreign body loosely implanted on the cornea into the lower fornix, from whence it can easily be removed. Rubbing the *affected* eye is, of course, more like to imbed the foreign body than to release it.

Should both these methods fail instil oil if much time is likely to elapse before expert medical attention is available, but this should always be secured if a feeling of discomfort persists, whether a foreign body can be seen or not.

When fragments of broken glass or splinters of metal may have entered or cut the eye, avoid any manipulation other than removal of very gross pieces, as more harm than good is done by unskilled attentions.

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